MHC CLASS II ANTIGEN PRESENTING SYSTEMS AND
METHODS FOR ACTIVATING CD4+ T CELLS

Cross-Reference to Related Applications

Insert 5   This application is a ~~continuation-in-part application of copending~~ United States provisional application Serial No. 60/018,175, filed May 23, 1996, having the same title as above, the disclosure of which are hereby incorporated by reference.

Technical Field

The present invention relates to materials and methods of activating CD4+ T cells with specificity for particular antigenic peptides, the use of activated T cells *in vivo* for the treatment of a variety of disease conditions, and compositions appropriate

15   for these uses.

Background

Though the T cell repertoire is largely shaped during T cell development in the thymus, mature CD4+ T cells are also

20   regulated extrathymically. Whereas, some conditions of activation lead to tolerance reflecting either anergy or clonal elimination, other conditions lead to a change in the type of response observed. For CD4+ T cells, this change in functional phenotype is largely a change in the pattern of cytokines

25   produced. Although CD4+ T cells that are subject to acute activation maintain the ability to produce multiple cytokines, T cells obtained under conditions of chronic stimulation frequently demonstrate a more restricted pattern of cytokine production. For example, T cell clones maintained by repeated

30   stimulation *in vitro* have defined two major functional categories of CD4+ T cells referred to as Th1 and Th2 type cells. Th1 type cells produce primarily interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor (TNF), all of

which are referred to as inflammatory cytokines. In contrast, Th2 type cells typically produce IL-4, IL-5, and IL-10 and are important for antibody production and for regulating the responses of Th1 type cells.

5   Although such extreme segregation in cytokine production is often not seen during _in vivo_ T cell responses, recovery from certain types of infections, such as _Leishmania_, is associated with preferential production of IL-2/IFN-γ. Mice that mount a Th2 response to _Leishmania_ fail to contain the infection and

10   ultimately die. Inappropriate production of cytokines of the Th2 type response has been frequently linked to allergic type diseases such as asthma and contact sensitivity. For review on activation of CD4⁺ T cells and role in allergic disease, see Hetzel and Lamb, _Clinical Immunol. Immunopath._, 73:1-10 (1994).

15

Perhaps the strongest association of human disease with skewed patterns of cytokine production is the association of Th1 responses and Th1 type cytokines with autoimmune disease. Strong evidence in experimental models indicates that many types

20   of autoimmunity including diabetes, experimental models for multiple sclerosis, autoimmune thyroiditis, and the like are mediated by Th1 type CD4⁺ T cells. The expression of Th2-associated cytokines, such as IL-4, in these models interfere with the development of autoimmune disease. Th2 type cytokines

25   dampen the response of Th1 type cells while the Th1 type cytokines antagonize the development of Th2 type responses.

In view of the association of particular activated T cell subsets with particular disease conditions, a need therefore exists to be able to direct the proliferation and activation of

30   CD4⁺ T cells to a desired T cells subset, a process that is extremely beneficial in altering the course of disease. One potential solution is to activate _in vitro_ CD4⁺ T cells that are first isolated from a subject who may optionally be having

either allergy or autoimmune conditions to produce cells secreting a preferred cytokine profile. The resultant activated T cells are then reintroduced to the subject to alter the course of disease and perhaps even provide a long term cure.

5   The challenge in this approach, now solved by the present invention, is the difficulty in defining activation conditions that reproducibly generate CD4+ T cell subsets that produce the desired therapeutic cytokine profile. Expression of particular cytokines is linked to a particular antigen presenting cell

10 (APC) and their associated accessory (assisting) molecules. For a review of the surface proteins serving as accessory molecules that are involved in T cell costimulation, see Mondino and Jenkins, <u>J. Leukocyte Biol.</u>, 55:805-815 (1994). Since both the cytokines produced by the APC and the coordinately expressed

15 accessory molecules are themselves regulated by multiple factors, including the type of antigen, the affinity of the T cell receptor (TCR)-antigen interaction, antigen concentration and the like, predicting the outcome of T cell activation upon antigen presentation is historically very difficult. Indeed, as

20 additional accessory molecules have been proposed for the activation process <u>in vivo</u>, it has become increasingly clear that many diverse molecules are involved in the regulation of T cell responses and act in combinatorial fashion to effect the outcome of T cell activation.

25   Prior to the present invention, the co-expression of selected MHC class II molecules in conjunction with one or more selected accessory molecules has not been possible. The present invention now presents a solution to predictably generate a preferred T cell phenotype through the reproducible activation

30 of T cells to generate either Th1 or Th2 type T cells. The invention describes the generation of synthetic APC that present, in a neutral background, MHC class II molecules in combination with defined accessory molecules. The MHC class II

molecules and defined accessory molecules are expressed in a
nonmammalian insect cell and can be presented in a variety of
forms of synthetic APC including insect cells displaying the
molecules.

5   The advantage of using the insect cells as the expression
and presentation vehicles for the MHC class II/accessory
molecule compositions of this invention is that the cells do not
endogenously produce regulatory cytokines and do not express
mammalian accessory molecules. This overcomes the inherent
10   unpredictability of using mammalian APC that express many
molecules that are capable of altering the T cell response. In
addition, the insect cell expression system described in the
present invention provides for the expression of MHC class II
molecules without bound peptide (i.e., "empty" molecules) that
15   can be produced under certain restrictive circumstances, such as
temperature requirements. At physiological temperatures, these
"empty" molecules are normally unable to reach the cell surface
as class II without bound peptide are very thermolabile. The
invention utilizes the capacity of "empty" MHC class II
20   compositions to allow for the exogenous loading of selected
peptides along with the ability to provided endogenously loaded
counterparts.

         A recombinant glycosyl-phosphatidylinositol (GPI)-modified
MHC class I molecule (HLA-A2.1:GPI/β_2m) was generated in the
25   above-described insect cell system to produce antigen presenting
cells as described in International Publication Number WO
96/12009 by Tykocinski. In that publication, the recombinant
GPI-modified MHC class I molecules are isolated from the insect
cell by affinity purification for subsequent reincorporation
30   into cell membranes. In other aspects, the publication
describes the preparation of a GPI-modified MHC class I molecule
co-anchored on a cell membrane with a GPI-modified B7.1
costimulatory molecule. Although the publication states that

GPI-modified MHC class II molecules can be prepared as described for those of MHC class I, the publication does not present any details for such preparation.

In contrast, the present invention provides and describes a unique means based on the co-expression of a selected MHC class II haplotype in conjunction with one or more accessory molecules, such as B7.1, to activate CD4$^+$ T cells resulting in the differentiation to a particular T cell subset, Th1 or Th2 cells, that effect a preferred cytokine profile influence. The invention provides the advantage of selectively activating CD4$^+$ T cells _in vitro_ to a preferred T cell subset thereafter allowing for the reintroduction of the activated T cells into the patient. The present invention thus provides the ability to combine individual presenting molecules with particular accessory molecules for expression in selected combinations that permits reproducibility and predictability for selectively activating CD4$^+$ T cells to a desired T cell subset not available in other approaches.

Brief Summary of the Invention

It has now been discovered that recombinant MHC class II molecules expressed in combination with selected accessory molecules, including costimulatory molecules and adhesion molecules, are effective in activating CD4$^+$ T cells to become armed effector T cells that recognize target cells on which MHC class II heterodimer is expressed for complexation with peptide. Activation is characterized by proliferation and differentiation into effector T cell subsets, Th1 and Th2, that secrete particular cytokines. Th1 and Th2 type T cells are respectively referred to as inflammatory cells and T-helper cells.

Thus, the present invention relates to a synthetic antigen presenting system, also referred to as APC, for producing and presenting a mammalian, preferably human, MHC class II molecule

in combination with one or more accessory molecules to activate CD4⁺ T cells.

In one embodiment, the system relates to a synthetic antigen presenting matrix having a support and at least the
5   extracellular portion of a MHC class II heterodimeric molecule operably linked to the support and capable of binding to a selected peptide. The matrix also includes an accessory molecule operably linked to the support. The accessory molecule interacts with a specific receptor present on the CD4⁺ T cell.
10 The MHC class II and accessory molecules are present in sufficient numbers to activate a population of CD4⁺ T cells specific for the MHC class II/peptide combination when the peptide is bound to the extracellular portion of the MHC molecule.

15   It has been found that an antigen presenting matrix having both a MHC class II heterodimer or at least the extracellular portion thereof loaded with a peptide specific for that MHC, together with an accessory molecule, provides a synergistic reaction in activating CD4⁺ T cells. Examples of accessory
20 molecules are costimulatory molecules, including B7.1 and B7.2, adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-3 (LFA-3), and survival molecules such as Fas ligand (FasL) and CD70. In some embodiments, the extracellular portion of such accessory
25 molecules can also be used in the present invention.

The support used for the matrix can take several different forms. Examples for the support include solid support such as metals or plastics, porous materials such as resin or modified cellulose columns, microbeads, microtiter plates, red blood
30 cells and liposomes.

Another type of support is a cell fragment, such as a cell membrane fragment. An entire cell is also contemplated as a support. In this embodiment, the matrix is actually cells which

have been transformed with one or more expression vectors containing genes for the expression of MHC class II α- and β-chains along with at least one accessory molecule. The expressed proteins are then transported to the cell membrane

5 where the transmembrane domain of the class II chains provide anchors allowing the extracellular domain to be displayed on the outer cell surface, thereby creating a synthetic antigen presenting cell (APC). The expression vectors contain the selected genes, preferably in the form of a cDNA sequence,

10 operably linked to a promoter that is either constitutive or inducible.

The MHC α- and β-chains associate together forming a MHC class II heterodimer which binds to a peptide specific for that heterodimer. With the present invention, two methods of loading

15 peptides onto MHC class II heterodimers are contemplated. In one embodiment, the peptide is loaded intracellularly following proteolytic processing of internalized intact protein into peptide fragments. The peptides are then loaded onto newly generated MHC class II molecules while they are still within the

20 cell. Alternatively, the MHC class II molecules are expressed as empty molecules on the cell surface and synthetic or processed peptide reagents are then loaded extracellularly onto the MHC class II heterodimer.

Nucleotide sequences for encoding at least one accessory

25 molecule gene operably linked to a promoter in a vector are also introduced into the cell. Following expression, the accessory molecule is coordinately anchored on the surface of the cell along with the MHC class II heterodimer in sufficient numbers to activate a population of CD4+ T cells lymphocytes specific for

30 the MHC class II/peptide complex. Other molecules referred to as antigen processing assisting molecules are also contemplated for use in generating recombinant APC. These molecules are either provided by the cell used as APC or exogenously through

an expression vector system as described above. Examples of such antigen processing assisting molecules include invariant chain, lysosomal enzymes and H2-M and H2-O molecules.

     The cell line is synthetic in that at least one of the

5   genes described above is not naturally present in the cells from which the cell line is derived. It is preferable to use a poikilotherm cell line because MHC molecules are thermolabile. A range of species are useful for this purpose. See, for example, U.S. Patent No. 5,314,813 to Petersen et al. which

10 discusses numerous species for this use, the disclosure of which is hereby incorporated by reference. Eukaryotic cells and preferably insect cells are used as APC. Preferred insect cells include Drosophila (fruit fly) and Spodoptera (butterfly).

     MHC class II molecules have been expressed in insect cells

15 such as Drosophila and Spodoptera cells. Since these cells do not have all the components of a mammalian immune system, the various proteins involved in the peptide loading machinery are absent from such cells. The lack of mammalian peptide-loading machinery allows the introduced mammalian MHC class II molecules

20 to be expressed as empty molecules at the cell surface when the cells are cultured at thermostabile temperature restrictive conditions, such as at 28°C. In contrast, at 37°C, empty Class I molecules are thermolabile and tend to disintegrate. Thus, by incubating MHC class II-expressing Drosophila cells with

25 peptides that specifically bind to anchored MHC class II molecule, virtually every class II molecule is loaded with one and the same peptide. Moreover, the invention provides for the means to introduce any known MHC class II α- and β-chain genes into an expression vector thereby overcoming the inherent limit

30 to the number of MHC class II molecules expressed in any one mammal.

     In the present invention, a specifically effective synergistic reaction in driving CD4⁺ T cells to a Th1-type

response characterized by an increase in the cytokine interleukin-2 (IL-2) results from a _Drosophila_ antigen presenting cell having MHC class II molecules bound with a peptide, a costimulatory molecule, and an adhesion molecule. In

5   particular, a highly effective synergistic generation of IL-2 production coupled with CD4+ proliferation results from the combination of B7.2 and ICAM-1. In contrast, without ICAM-1 but with either B7.1 or B7.2, the _Drosophila_ APC system loaded with peptide induced a Th2-type response characterized by an increase

10   in IL-4. Thus, ICAM-1 antagonized the Th2-type response resulting in a Th1-type phenotype.

A Th1 phenotype characterized by IL-2 production coupled with proliferative responses also resulted from a synthetic antigen presenting cell having CD70 expressed simultaneously

15   with ICAM-1 with or without B7.2.

Therefore, the selection of MHC class II genes in combination with at least one accessory molecule genes for expression thereof in an APC of this invention can be tailored depending upon the desired outcome for effecting proliferation

20   and phenotypic activation of CD4+ T cells.

The present invention also relates to methods for making the synthetic APC systems as described above in which at least one expression vector containing genes for a MHC class II heterodimer and an accessory molecule is introduced.

25   Methods of producing activated CD4+ T cells _in vitro_ are also contemplated. One preferred method comprises contacting, _in vitro_, CD4+ cells with a synthetic MHC class II/accessory molecule-bearing APC described above for a time period sufficient to activate, in an antigen-specific manner, a

30   population of CD4+ T cells. The method may further comprise (1) separating the activated CD4+ cells from the antigen-presenting matrix; (2) suspending the activated CD4+ cells in an acceptable carrier or excipient; and (3) administering the suspension to an

individual in need of treatment. As previously discussed, the antigens may comprise native or undegraded proteins or polypeptides, or they may comprise antigenic polypeptides which have been cleaved or synthesized into peptide fragments

5 comprising at least 8 amino acid residues prior to incubation with the mammalian MHC class II heterodimeric molecules.

In addition to the utility of being able to direct the activation of CD4⁺ T cells to a desired T cell subset as described above, the ability to express any MHC class II

10 molecule provides the means to identify unknown CD4⁺-activating peptide specific for that particular MHC class II molecule. As such, the present invention contemplates the activation of CD4⁺ T cells through the screening of a peptide library with synthetic APC expressing a particular MHC class II heterodimer.

15 In a further embodiment, the synthetic APC system described herein is useful for isolation of reactive CD4⁺ T cells from a heterologous population of cells. Such isolation provides the ability to monitor ongoing CD4⁺ T cell-mediated responses in disease conditions in a patient.

20 In another variation of the above, in view of the ability to selectively activate CD4⁺ T cells into a particular T cell subset for producing a preferred cytokine profile; the invention relates to methods of treating conditions in patients mediated by a undesirable CD4⁺ response. Such disease conditions

25 characterized by either a Th1- or Th2-type response include autoimmune diseases, allergy and cancer. The therapeutic goal is to introduce CD4⁺ T cells activated to a preferred T cell subset to antagonize an ongoing CD4⁺ T cell response. Thus, the method comprises (1) obtaining a fluid sample containing resting

30 or naive CD4⁺ cells from the patient; (2) contacting, in vitro, the CD4⁺ cells with a selected synthetic peptide-loaded APC of this invention for a time period sufficient to activate, in an

antigen-specific manner, the CD4⁺ cells; and (3) administering
the activated CD4⁺ cells to the patient.

Other embodiments are apparent to one skilled in the art.

5 Brief Description of the Drawings

Figures 1A-1C diagram the construction of expression
plasmids pRmHa-2 and pRmHa-3. In Figure 1A, pRmHa-2
construction is shown; in Figure 1B, pRmHa-3 construction is
shown; and in Figure 1C, the pRmHa-3 vector is illustrated,
10 showing the restriction, polylinker, promoter, and
polyadenylation sites, as well as a site at which a nucleotide
sequence may be inserted for expression.

Figure 2 shows the proliferative response of DO10 T cell
receptor (TCR) transgenic mouce cell line (Tg cells) when
15 cultured in the presence of _Drosophila_ cell lines with or
without ovalbumin (OVA) peptide loaded onto the surface-
expressed MHC class II heterodimer. Proliferation is assayed as
described in Example 5. The proliferation is measured in counts
per minute (cpm) X 1000 as plotted on the Y-axis against
20 increasing concentrations of OVA peptide in μM on the X-axis.
T-S (diamond marked line) shows responses with control splenic
APC. Proliferative responses with recombinant MHC class II
alone are shown in the line having closed circles. Those with
MHC class II combined with either costimulatory molecules B7.1
25 or B7.2 are shown respectively with lines having open and closed
squares.

Figure 3 shows proliferative responses to recombinant MHC
class II alone (closed circle line), with MHC class II plus B7.2
(closed square line), with MHC class II plus ICAM-1 (open
30 triangle line) and with MHC class II plus B7.2 and ICAM-1 (open
diamond line). Refer to Figure 2 legend for other details.

Figures 4A-4D show the cytokine profile produced in
response to activation of CD4⁺ T cells when cultured in the

presence of <u>Drosophila</u> APC having recombinant MHC class II alone or in combination with B7.1 or B7.2 costimulatory molecules. Splenic APC (labeled T-S) are control assays. The assays are performed as described in Example 5. Figures 4A-4D respectively

5 show the cytokines Il-2, Il-4, IFN-γ and Il-10 in ng/ml as plotted on the Y-axis. Cytokine profiles were assessed over three days between day 3 and 5 of culture.

Figures 5A-5D show the cytokine profile produced in response to activation of CD4⁺ T cells when cultured in the

10 presence of <u>Drosophila</u> APC having recombinant MHC class II alone or in combination with B7.2 costimulatory molecule, ICAM-1 and with B7.2 and ICAM-1. Figures 5A-5D respectively show the cytokines Il-2, Il-4, IFN-γ and Il-10 in ng/ml as plotted on the Y-axis. Refer to Figure 4 legend for other details.

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<u>Detailed Description of the Invention</u>

A.   <u>Definitions</u>

<u>Amino Acid Residue</u>: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide

20 linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH₂ refers to the free amino group present

25 at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in <u>J. Biol. Chem.</u>, 243:3552-3559 (1969) and adopted at 37 CFR §1.822(b)(2)), abbreviations for amino acid residues are shown

30 in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by formulae have a left- to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as

those listed in 37 CFR 1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid

5 residues or a covalent bond to an amino-terminal group such as NH₂ or acetyl or to a carboxy-terminal group such as COOH.

Recombinant DNA (rDNA) molecule: A DNA molecule produced by operatively linking two DNA segments. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two

10 nucleotide sequences not normally found together in nature. rDNA's not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

Vector: A rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or

15 polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to herein as "expression vectors".

Upstream: In the direction opposite to the direction of

20 DNA transcription, and therefore going from 5' to 3' on the non-coding strand, or 3' to 5' on the mRNA.

Downstream: Further along a DNA sequence in the direction of sequence transcription or read out, that is traveling in a 3'- to 5'-direction along the non-coding strand of the DNA or

25 5'- to 3'-direction along the RNA transcript.

Reading Frame: A particular sequence of contiguous nucleotide triplets (codons) employed in translation that define the structural protein encoding-portion of a gene, or structural gene. The reading frame depends on the location of the

30 translation initiation codon.

Polypeptide: A linear series of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues.

Protein: A linear series of greater than 50 amino acid residues connected one to the other as in a polypeptide.

Receptor: A receptor is a molecule, such as a protein, glycoprotein and the like, that can specifically (non-randomly) bind to another molecule.

Substantially Purified or Isolated: When used in the context of polypeptides or proteins, the terms describe those molecules that have been separated from components that naturally accompany them. Typically, a monomeric protein is substantially pure when at least about 60% to 75% of a sample exhibits a single polypeptide backbone. Minor variants or chemical modifications typically share the same polypeptide sequence. A substantially purified protein will typically comprise over about 85% to 90% of a protein sample, more usually about 95%, and preferably will be over about 99% pure. Protein or polypeptide purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a sample, followed by visualization thereof by staining. For certain purposes, high resolution is needed and high performance liquid chromatography (HPLC) or a similar means for purification utilized.

Synthetic Peptide: A chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

B.   MHC Class II Heterodimers, Accessory Molecules and Antigen Processing Assisting Molecules

The present invention relates to a synthetic antigen-presenting system for use in activating CD4+ T cells. Though the T cell repertoire is largely shaped during T cell development in the thymus, mature CD4+ T cells are also regulated extrathymically. Although some conditions of activation lead to tolerance reflecting either anergy or clonal elimination, other

conditions lead to a change in the type of response observed.
For CD4$^+$ T cells, this change in functional phenotype is largely
a change in the pattern of cytokines produced.   Although CD4$^+$ T
cells that are subject to acute activation maintain the ability
5   to produce multiple cytokines, T cells obtained under conditions
of chronic stimulation frequently demonstrate a more restricted
pattern of cytokine production.   For example, T cell clones
maintained by repeated stimulation in vitro have defined two
major functional categories of CD4$^+$ T cells referred to as Th1
10   and Th2 type cells.   Th1 type cells produce primarily
interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis
factor (TNF), all of which are referred to as inflammatory
cytokines.   As such, the TH1 cells are sometimes referred to as
inflammatory T cells which then activate macrophages to kill
15   intravesicular pathogens they harbor as mediated by peptides
generated from processing of pathogenic proteins presented to
CD4$^+$ T cells.   In contrast, Th2 type cells typically produce
IL-4, IL-5, and IL-10 and are important for antibody production
and for regulating the responses of Th1 type cells.   As such,
20   Th2 cells are sometimes referred to as helper T cells that
activate B cells to make antibody in response to peptides
derived from extracellular pathogens and toxins that are
presented to CD4$^+$ T cells.

A CD4$^+$ T cell is defined as a T cell or a T lymphocyte that
25   has the CD4$^+$ co-receptor on its cell surface in conjunction with
the presence of either α:β or γ:Δ heterodimeric receptor
associated with the proteins of the CD3 complex.

Activation of CD4$^+$ T cell subsets is characterized by
proliferation of the responsive T cell population coordinated
30   with the selection production of cytokines as described above
dependent upon the type of stimulation induced by an antigen
presenting cell.   The latter is defined as a highly specialized
cell that can process antigens and display their peptide

fragments on the cell surface together with molecules required
for lymphocyte activation. The specificity of CD4+ T cell
activation is based on the T cell antigen receptor (TCR)
recognition of peptide antigens bound to MHC class II
5   heterodimers on the surface of antigen presenting cells (APC).
The main APC for T cells are dendritic cells, macrophages and B
cells. In addition, APC-derived non-antigenic costimulatory
signals play a contributory role in CD4+ T cell activation.

The present invention utilizes a synthetic antigen
10   presenting system, based on the natural immunological response
mechanisms described above, to manipulate the activation of CD4+
T cells by recombinant MHC class II molecules in conjunction
with one or more accessory molecules broadly referred to as
costimulatory molecules. The latter include specific
15   costimulatory molecules, adhesion molecules and survival
molecules, and the like. In other aspects of the invention, the
synthetic antigen presenting system further contains antigen
processing assisting molecules that are useful in generating
peptide-loaded MHC class II molecules. In the context of this
20   invention, the synthetic antigen presenting systems are useful
for activating CD4+ T cells _in vitro_ and _in vivo_. These aspects
are discussed in Section E on Methods of Altering CD4+ T Cell
Responses.

Thus, as mentioned above, the synthetic antigen presenting
25   system of the present invention has at least two major
components. The first component is at least the extracellular
portions of a recombinant MHC class II heterodimer which is
capable of binding to a peptide that provides the specificity of
CD4+ T cell activation via recognition by the TCR. The second
30   major component is at least the extracellular portion of at
least one accessory molecule that provides a non-antigenic
specific costimulatory signal in the activation of CD4+ T cells.
In other embodiments, the entire molecules of the antigen

presenting system and the non-antigen costimulatory signals are
used.

   For ease of description, MHC class II heterodimers will be
discussed generally, with the understanding that an
extracellular portion of the MHC molecule may be used in certain
aspects of the invention. The portion of the MHC molecule
necessary for the present invention is the part that binds to
the antigenic peptide for presentation to the CD4$^+$ T cells.

   The present invention allows the recombinant MHC class II
heterodimers to be produced by vector-transformed synthetic
antigen presenting cells with the peptide already complexed with
the MHC class II heterodimer. Alternatively, empty MHC class II
heterodimers are produced that do not yet have a peptide
complexed with them. This latter embodiment is particularly
useful as it allows for complexation with a particular peptide
or for screening of a library of peptides after the MHC class II
heterodimers are expressed.

   1.   MHC Class II Genes and Encoded Heterodimers

        MHC class II molecules are cell surface glycoproteins
that consist of a non-covalent complex of two chains, α and β,
both of which span the membrane. A peptide-binding cleft is
formed between the cooperating chains. Peptides that bind MHC
class II molecules are variable in length and have anchor
residues that lie at various distances from the ends of the
peptides thereby resulting in peptides having ends that are not
tightly bound within the cleft of the binding pocket. See,
Janeway and Travers, Immunobiology, Section 4-4, Current Biology
LTD, 2nd ed., 1996. Further aspects of the presenting antigenic
peptides are discussed in Section D.

   In vivo, empty MHC class II heterodimers become
destabilized and are subsequently removed from the cell surface
thereby preventing MHC molecules for acquiring peptides from the

surrounding extracellular fluid that would deleteriously effect
T cell specificity. In present invention, the synthetic antigen
presenting cell system allows for the production of empty MHC
class II heterodimers on the cell surface that are not subject
5   to destabilizing events. As a consequence, loading of an
antigenic peptide on a surface-expressed recombinant MHC class
II heterodimer is facilitated for subsequent use in manipulating
CD4⁺ activation and cytokine production.

The present invention, particular combinations of a
10 selected MHC class II molecule with a coordinate antigen is
facilitated by the presence of consensus nucleotide sequences in
MHC class II genes. These regions, described further below,
allow for the retrieval and use of the multiple MHC class II
genes in the MHC complex in mammals as well as the multiple
15 alleles of each gene. In other words, MHC is both polygenic and
polymorphic having respectively several genes and multiple
alleles of each gene.

In humans, MHC is called HLA while in mouse it is referred
to as H-2. Three pairs of MHC class II α- and β-chain genes in
20 humans have been designated HLA-DP, HLA-DQ and HLA-DR. The HLA-
DR cluster contains an extra β-chain gene. As such, the three
sets of genes can give rise to four types of MHC class II
molecules. MHC class II genes and encoded α- and β-chains that
are obtained from human genes are said to be of human origin.
25 In mice, MHC class genes are designated H2-M, H2-A and H2-E. As
each MHC class II molecule binds a different range of peptides,
the presence of multiple gene loci gives an individual the
ability to present a broad range of different peptides than if
only one MHC class II molecules of each class were expressed at
30 the cell surface.

While the polymorphic MHC class II genes encode
corresponding proteins that vary by only one or a few amino
acids, the different allelic variants differ by up to 20 amino

acids. As a result, MHC class II diversity expands the ability of antigen recognition by T cells. Moreover, via MHC restriction, T cells have been shown to recognize peptide in the context of a particular MHC molecule but not when presented on

5   another. Thus, T cell receptor specificity is imparted by both peptide and by the MHC molecule binding it.

The MHC class II heterodimers of this invention containing selected α- and β-chains are obtained by amplification of MHC class II-encoding genes and allelic variants thereof with

10 specific pairs of oligonucleotide primers. The nucleotide sequences of the primers allow for amplification of the diversity of MHC class II genes and allelic variants thereof based on the 5' and 3' consensus nucleotide sequences present in the genes within a category of genes. Specific nucleotide

15 sequences of primer pairs for amplifying the α- and β-chains of human HLA-DP, -DQ and -DR genes as well as those for amplifying murine IA^d-encoding heterodimers are presented in Example 2A.

The MHC class II-encoding genes are amplifiable from a variety of cellular sources including B cells, macrophages and

20 dendritic cells, all of which are present in the blood. The amplification conditions for obtaining amplified MHC class II-encoding genes with the primers of this invention are described in Section C.

The α- and β-chains comprising the MHC heterodimers of this

25 invention are useful in either anchored or soluble form. In the anchored form, the recombinant MHC heterodimer is anchored into the synthetic antigen presenting cell from which it is expressed. Alternatively, a recombinant MHC heterodimer is anchored in a matrix comprising a support as described in this

30 invention after being secreted in soluble form. The latter is generated when a stop codon is engineered during the amplification procedure or thereafter into the nucleotide

sequence encoding the MHC class II α- and β-chains of choice
preceding the transmembrane domain.

2.   Accessory Genes and Encoded Molecules

5   The accessory molecules of this invention, including
costimulatory molecules, adhesion molecules and survival
molecules, are effective in concert with the MHC class II
heterodimer complexed with peptide in activating CD4+ T cells to
become armed effector T cells that recognize target cells.

10 Naive T cells are activated to proliferate and differentiate
into armed effector T cells when they encounter their specific
antigen when presented by a peptide-loaded MHC class II
heterodimer on the surface of an APC. Activation not only
requires the recognition of a foreign peptide fragment bound to

15 a MHC class II heterodimer but it also requires the simultaneous
delivery of a costimulatory signal concurrently expressed by the
APC.

          Thus, the synthetic APC or matrices of this invention are
characterized by the presence not only of a particular MHC class

20 II heterodimer but also by the presence of one or more
costimulatory molecules that are broadly defined as accessory
molecules. At least three types of accessory molecules,
including specific costimulatory molecules, adhesion molecules,
and survival molecules, are contemplated for use in preparing

25 synthetic APC or matrices of this invention

a.   Costimulatory Molecules

          A first type of an accessory molecule is composed
of costimulatory molecules such as B7.1 (previously known as B7

30 and also known as CD80) and B7.2 (also known as CD86) which
binds to CD28 on T cells. B7.1 and B7.2 are structurally
related glycoproteins that are homodimeric members of the
immunoglobulin superfamily. Other costimulatory molecules are

anti-CD28 antibodies or the functional portions of such
antibodies, e.g. Fab portions that bind to CD28. Ligation of
CD28 by the above molecules has been shown to costimulate the
growth of naive T cells. On activated T cells, an additional

5 receptor, CTLA-4, binds B7 molecules with a higher affinity that
that with CD28.

          Recombinant B7 costimulatory molecules for use in the
synthetic APC or matrices of this invention are obtained by PCR
as described for MHC class II molecules. Preferred

10 oligonucleotide primers and cellular sources for amplification
therefrom as described in Example 2C.

          b.   Adhesion Molecules

                Another major type of accessory molecule of the
15 present invention is an adhesion molecule that also functions in
T cell activation. Accesory adhesion molecules include the
various ICAM molecules, which include intercellular adhesion
molecule (ICAM) ICAM-1, ICAM-2, ICAM-3, lymphocyte function-
associated antigen (LFA) LFA-1 and LFA-3. All of these

20 molecules are members of the immunoglobulin superfamily. The
ICAM-related members all bind to the T cell integrin, LFA-1. In
addition to being expressed on APC including dendritic cells,
macrophages and B cells, ICAM-1 and ICAM-2 are also expressed on
endothelium, thereby mediating cell adhesion and subsequenct

25 extravasation between circulating leukocytes and endothelium.
ICAM-3, however, is only expressed on leukocytes and is thought
to play an important part in adhesion between T cells and APC.

          The interaction between ICAM-1, -2 and -3 synergizes with a
second adhesive interaction between LFA-3 (CD58) and LFA-2 (CD2)

30 that are respectively expressed on an APC and a T cell surface.

          Recombinant adhesion molecules for use in the synthetic APC
or matrices of this invention are obtained by PCR as described

for MHC class II molecules. Preferred oligonucleotide primers
and cellular sources for amplification therefrom as described in
Example 2C.

5   c.   <u>Survival Molecules</u>

A survival molecule is another type of an
accessory molecule that plays a role in metabolic responses
ranging from stimulatory to inducing cell death. Thus, a
survival molecule can also be referred to as a cell death
10 regulating molecule. A survival molecule is typically a protein
but may include other types of macromolecules such as
carbohydrates, lipids and the like. Survival molecules for use
in the compositions and methods of this invention include Fas
ligand, TNF-receptor, TNF, CD70, a Type II transmembrane protein
15 that is a member of the TNF family that binds to CD27, a member
of the TNF receptor family. Fas ligand binds to the receptor
called Fas and receptor occupancy resulting in the induction of
apoptotic cell death of the cell expressing Fas receptor. CD27
is expressed on resting T and B cells while CD70 is expressed on
20 activated T and B cells. Binding of CD70 to its receptor, CD27,
induces T-cell costimulation and the interaction may be
important for the recruitment of T cells from the unprimed T
cell pool. Under other certain conditions, activation of the
TNF receptor by TNF results in a similar response.
25   The recombinant survival molecules described above for use
in the synthetic APC or matrices of this invention are obtained
by PCR as described for MHC class II molecules. Preferred
oligonucleotide primers and cellular sources for amplification
therefrom as described in Example 2C.
30   As shown in the Examples, particular combinations of a
peptide bound to a recombinant MHC class II molecule used in
conjunction with one or more of the above-described recombinant
accessory molecules activates T cells into armed effector T

cells that are distinguishable into Th1 inflammatory T cells and Th2 helper T cells.

3. Antigen Processing Assisting Genes and Encoded Molecules

a. HLA-DM

HLA-DM in humans and H2-M in mice is a MHC class II-like molecule that is also encoded within the MHC class II gene clusters. HLA-DM, like MHC class II, contains both α- and β-chain genes forming a heterodimer. However, unlike MHC class II molecules, peptide loading is not required for stabilizing the molecule. HLA-DM facilitates the loading of peptides onto newly formed MHC class II heterodimers following the removal of the invariant chain as further described below. Recombinant HLA-DM is contemplated for use in the compositions and methods of this invention to assist in the loading of internally processed peptides.

b. Invariant Chain

The invariant chain is a specialized protein that binds to newly formed MHC class II heterodimers thereby forming a trimer with each subunit of the MHC class II heterodimer. The trimerized molecule prevents the loading of intracellular peptides present in the endoplasmic reticulum but it also facilitates the export of the molecule from that compartment. Thereafter, the invariant chain is cleaved through multiple steps resulting in a MHC class II heterodimer that can then be complexed with processed peptides.

Thus, recombinant invariant chain is contemplated for use in the compositions and methods of this invention to assist in the loading of internally processed peptides.

C.   Nucleic Acids and Polynucleotides

1.   PCR to Obtain Genes Encoding MHC Class II and
      Accessory Molecules

Nucleic acid sequences encoding MHC class II
molecules, accessory molecules and antigen processing assisting
molecules of this invention are obtained in a number of ways
familiar to one of ordinary skill in the art including direct
synthesis, cloning, purification of DNA from cells containing
such genes, and the like. One expedient means to obtain genes
for encoding the molecules used in the compositions and methods
described herein is by polymerase chain reaction (PCR)
amplification on selected nucleic acid templates with selected
oligonucleotide primer pairs as further described below.

Known, partial and putative human leukocyte antigen (HLA),
the genetic designation for the human MHC, amino acid and
nucleotide sequences, including the consensus sequence, are
published (see, e.g., Zemmour and Parham, Immunogenetics 33:
310-320 (1991)), and cell lines expressing HLA variants are
known and generally available as well, many from the American
Type Culture Collection ("ATCC"). Therefore, using PCR, MHC
class II-encoding nucleotide sequences are readily operatively
linked to an expression vector of this invention that is then
used to transform an appropriate cell for expression therein.

Particularly preferred methods for producing the
recombinant molecules of the present invention rely on the use
of preselected oligonucleotides as primers in PCR to form PCR
reaction products as described herein.

If a gene is to be obtained by PCR amplification, in
general, two primers comprising a PCR primer pair, are used for
each strand of nucleic acid to be amplified. For the sake of
simplicity, synthesis of exemplary MHC class II heterodimer-
encoding genes is discussed, but it is expressly to be
understood that the PCR amplification method described is

equally applicable to the synthesis of MHC class II allelic variants, accessory molecules and antigen processing assisting molecules, including those whose complete sequences are presently unknown.

5   In general, a first primer is referred to a a forward primer or a 5' primer as it has the same sequence as the top strand of template DNA and thus hybridizes to the bottom complementary strand.

A second primer is referred to as a backward primer or a 3'
10   primer as it has the same sequence as the bottom strand and thus hybridizes to the complementary sequence on the top strand. Typically, in other words, one primer is complementary to the negative (-) or bottom strand of the nucleotide sequence and the other is complementary to the positive (+) or top strand.

15   In preferred aspects, both first and second primers are chosen to hybridize to (i.e., be complementary to) conserved regions within the MHC class II genes. However, primers can be designed to amplify specific MHC class II genes and allelic variants thereof by hybridizing to unique rather than consensus
20   sequences. For this aspect, the template sequence is preferably known for design of such primer pairs.

One or both of the first and second primers can be designed to introduce into the amplified product a nucleotide sequence defining an endonuclease recognition site. The site can be
25   heterologous to the MHC class II gene being amplified and typically appears at or near the 5' end of the primer. It may also be helpful to place a 4-base spacer sequence proximal to the restriction site to improve the efficiency of cutting amplification products with enzymes.

30   The primers of the invention for isolating specific nucleotide sequences include oligonucleotides of sufficient length and appropriate sequence so as to provide specific initiation of polymerization on a significant number of nucleic
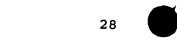
acids with the corresponding nucleotide sequence. Specifically, the term oligonucleotide primer as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and more preferably

5   around 20, the sequence of which is capable of initiating synthesis of a primer extension product.

Experimental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization and extension, such as thermostable polymerases,

10   and a suitable buffer, temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate the two strands before being used to prepare extension products. Preferably, the

15   primer is an oligodeoxyribonucleotide. The primer must be sufficiently long and substantially complementary to prime the synthesis of extension products in the presence of the inducing agent for polymerization and extension of the nucleotides. The exact length of primer will depend on many factors, including

20   temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 15-22 or more nucleotides, although it may contain fewer nucleotides. Alternatively, as is well known in the art, the mixture of nucleoside triphosphates can be biased to influence the

25   formation of mutations to obtain a library of mutated recombinant MHC class II-encoding molecules for use in presenting unique peptides to CD4$^+$ T cells.

Oligonucleotide primers of the invention are employed in the PCR amplification process which is an enzymatic chain

30   reaction that produces exponentially growing quantities of a nucleotide sequence. Annealing the primers to denatured nucleic acids followed by extension with a thermostable polymerase such as _Thermophilus aquaticus_ (_Taq_) and _Pyrococcus furiosus_ (_Pfu_)

(Hoffman La-Roche, Basal, Switzerland), and nucleotides, results in newly synthesized (+) and (-) strands. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in

5 exponential production of the DNA fragment defined by the primers. The product of the chain reaction is a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed. Those of skill in the art will know of other amplification methodologies which can also be

10 utilized to increase the copy number of target nucleic acids. These may include for example, ligation activated transcription (LAT), ligase chain reaction (LCR), and strand displacement activation (SDA), although PCR is the preferred method as described in the US Patents listed below.

15   The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods as described above for synthesis of complementary oligonucleotides or automated embodiments thereof. One method for synthesizing

20 oligonucleotides on a modified solid support is described in US Patent 4,458,066.

    Preferred primers for amplifying MHC class II genes, accessory molecule genes, and antigen processing assisting molecule genes are described in Example 2.

25   PCR amplification methods are described in detail in U.S. Patent Nos. 4,683,192, 4,683,202, 4,800,159, 4,965,188 and 5,395,750, the disclosures of which are hereby incorporated by reference, and at least in several texts including "PCR Technology: Principles and Applications for DNA Amplification",

30 H. Erlich, ed., Stockton Press, New York (1989); and "PCR Protocols: A Guide to Methods and Applications", Innis et al., eds., Academic Press, San Diego, California (1990). Various preferred methods and primers used herein are described

hereinafter and are also described by Zemmour, et al.,
Immunogenetics, 33:310-20 (1991), by Ausebel, et al., In Current
Protocols in Molecular Biology, Wiley and Sons, New York (1993)
and by Sambrook et al., Molecular Cloning: A Laboratory Manual,

5 Cold Spring Harbor Laboratory, (1989). Particular PCR methods
including nested PCR, overlap PCR, reverse-transcriptase-PCR,
and the like that are well known to one of ordinary skill in the
art are contemplated for use in obtaining the recombinant
molecules of this invention.

10   In alternative embodiments, the PCR process is used not
only to produce a variety of MHC class II-encoding molecules,
but also to induce mutations which may emulate those observed in
the highly-polymorphic MHC loci, or to create diversity from a
single parental clone and thereby provide a MHC class II-

15 encoding DNA "library" having a greater heterogeneity.

    2.   Expression Vectors

        The present invention contemplates plasmid expression
vectors in substantially pure form capable of directing

20 expression of MHC class II-encoding genes, accessory molecule
genes and antigen processing assisting genes to produce the
corresponding recombinant proteins. For simplicity, the above
genes are herein referred to collectively as polypeptide-
encoding nucleotide sequences. Vectors capable of directing the

25 expression of genes to which they are operatively linked are
referred to herein as "expression vectors" or "expression
plasmids", both of which are also referred to as "plasmids".

        As used herein, the term "vector" or "plasmid" refers to a
nucleic acid molecule capable of transporting between different

30 genetic environments another nucleic acid to which it has been
operatively linked. Preferred vectors are those capable of
autonomous replication and expression of structural gene
products present in the DNA segments to which they are

operatively linked. Vectors, therefore, preferably contain the replicons and selectable markers that are compatible with the host selection system. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal
5   replication.

A plasmid of this invention is a circular double-stranded plasmid that contains at least a regulation region having elements capable of activating transcription of the translatable polypeptide-encoding nucleotide sequences of this invention.
10   The plasmid further contains a translatable nucleotide sequence from which the desired encoded polypeptides of this invention are expressed. Thus, the vectors are said to be capable of directing the expression of the recombinant polypeptides described herein as encoded from the corresponding expressible
15   genes.

A preferred vector for use according to the present invention is a plasmid; more preferably, it is a high copy number plasmid. It is also preferable that the vector of choice be best suited for expression in the chosen host.
20   Such expression vectors contain a promotor sequence in the regulatory region which facilitates the efficient transcription of an inserted genetic sequence in the host. Preferably, the vector contain an inducible promoter sequence, as inducible promoters tend to limit selection pressure against cells into
25   which such vectors (which are often constructed to carry non-native or chimeric nucleotide sequences) have been introduced. The expression vector also typically contains an origin of replication as well as specific genes which allow phenotypic selection of the transformed cells. The DNA segment
30   can be present in the vector operatively (also referred to as operably) linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters).

In a separate embodiment, a plasmid also contains a gene, the expression of which confers a selective advantage, such as a drug resistance, to a host cell when introduced or transformed into that cell. Typical prokaryotic and eukaryotic drug

5   resistance genes respectively confer resistance to ampicillin or tetracyclin and to neomycin (G418 or Geneticin). Other drug resistance markers include chloramphenicol, kanamycin, streptomycin, carbenicillin, mercury, rifampcin, rifampicin, fusaric acid, and the like.

10   The choice of vector to which the regulatory region and nucleotide sequences for encoding polypeptides of the present invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., replication or protein expression, and the host cell to be

15   transformed, these being limitations inherit in the art of constructing recombinant DNA molecules.

Operatively linking refers to the covalent joining of nucleotide sequences, preferably by conventional phosphodiester bonds, into one strand of DNA, whether in single or double

20   stranded form. Moreover, the joining of nucleotide sequences results in the joining of functional elements such as response elements in regulatory regions with promoters and downstream polypeptide-encoding nucleotide sequences as described herein.

One typical method for operatively linking inserts into

25   expression plasmids is by directional ligation. This is accomplished through a sequence of nucleotides that are adapted for directional ligation. Such a sequence is referred to commonly as a polylinker that is a region of the DNA expression vector that (1) operatively links for replication and transport

30   the upstream and downstream translatable DNA sequences and (2) provides a site or means for directional ligation of a DNA sequence into the vector. Typically, a directional polylinker is a sequence of nucleotides that defines two or more

restriction endonuclease recognition sequences, or restriction
sites. Upon restriction cleavage, the two sites yield cohesive
termini to which a translatable DNA sequence can be ligated to
the DNA expression vector. Preferably, the two restriction

5   sites provide, upon restriction cleavage, cohesive termini that
are non-complementary and thereby permit directional insertion
of a translatable DNA sequence into the cassette.

     A variety of host-expression vector systems may be utilized
to express a polypeptide encoded by a nucleotide sequence.

10   These include but are not limited to microorganisms such as
bacteria transformed with recombinant bacteriophage DNA, plasmid
DNA or cosmid DNA expression vectors containing a polypeptide-
encoding nucleotide sequence; yeast transformed with recombinant
yeast expression vectors containing a polypeptide-encoding

15   nucleotide sequence; plant cell systems infected with
recombinant virus expression vectors (e.g., cauliflower mosaic
virus, CaMV; tobacco mosaic virus, TMV) or transformed with
recombinant plasmid expression vectors (e.g., Ti plasmid)
containing a polypeptide-encoding nucleotide sequence; insect

20   cell systems infected with recombinant virus expression vectors
(e.g., baculovirus) containing a polypeptide-encoding nucleotide
sequence; or animal cell systems infected with recombinant virus
expression vectors (e.g., retroviruses, adenovirus, vaccinia
virus) containing a polypeptide-encoding nucleotide sequence, or

25   transformed animal cell systems engineered for stable
expression. In such cases where glycosylation may be important,
expression systems that provide for translational and
post-translational modifications may be used; e.g., mammalian,
insect, yeast or plant expression systems.

30   Any of these systems are useful in practicing the methods
of this invention. With any of the above expression systems,
the selected host is then used for expression of at least one
MHC class II heterodimer alone or in conjunction with at least

one accessory molecule further with or without an antigen

processing accessory molecule depending on the actual mechanism

for loading peptides, i.e., internally or externally.

Depending on the host/vector system utilized, any of a

5   number of suitable transcription and translation elements,

including constitutive and inducible promoters, transcription

enhancer elements, transcription terminators, etc. may be used

in the expression vector (see e.g., Bitter, et al., Methods in

Enzymology, 153:516-544, (1987)). For example, when cloning in

10 bacterial systems, inducible promoters such as Pl of

bacteriophage λ, Plac, Ptrp, Ptac (Ptrp-lac hybrid promoter) and

the like may be used. When cloning in mammalian cell systems,

promoters derived from the genome of mammalian cells (e.g.,

metallothionein promoter) or from mammalian viruses (e.g., the

15 retrovirus long terminal repeat; the adenovirus late promoter;

the vaccinia virus 7.5K promoter) may be used. Promoters

produced by recombinant DNA or synthetic techniques may also be

used to provide for transcription of a polypeptide-encoding

nucleotide sequence.

20   In bacterial systems a number of expression vectors may be

advantageously selected for expression of a polypeptide-encoding

nucleotide sequence according to the methods of this invention.

For example, when large quantities are to be produced, vectors

which direct the expression of high levels of fusion protein

25 products that are readily purified may be desirable. Those

which are engineered to contain a cleavage site to aid in

recovering the protein are preferred. Such vectors include but

are not limited to the E. coli expression vector pUR278 (Ruther,

et al., EMBO J., 2:1791, (1983)), in which the polypeptide-

30 encoding nucleotide sequence may be ligated into the vector in

frame with the LacZ coding region so that a hybrid

polypeptide-LacZ protein is produced; pIN vectors (Inouye &

Inouye, <u>Nuc. Acids Res.</u>, 13:3101-3109, (1985); Van Heeke &

Schuster, <u>J. Biol. Chem.</u>, 264:5503-5509, (1989)); and the like.

In one embodiment, the vector utilized includes prokaryotic

sequences that facilitate the propagation of the vector in

5   bacteria, i.e., a DNA sequence having the ability to direct

autonomous replication and maintenance of the recombinant DNA

molecule extra-chromosomally when introduced into a bacterial

host cell. Such replicons are well known in the art.

Those vectors that include a prokaryotic replicon also

10 typically include convenient restriction sites for insertion of

a recombinant DNA molecule of the present invention. Typical of

such vector plasmids are pUC8, pUC9, pBR322, and pBR329

available from BioRad Laboratories, (Richmond, CA) and pPL

available from Pharmacia, (Piscataway, NJ), and pBLUESCRIPT and

15 pBS available from Stratagene, (La Jolla, CA). A vector of the

present invention may also be a Lambda phage vector including

those Lambda vectors described in <u>Molecular Cloning: A

Laboratory Manual</u>, Second Edition, Maniatis et al., eds., Cold

Spring Harbor, NY (1989).

20   In another preferred embodiment, plasmid vectors for use in

the present invention are also compatible with eukaryotic cells.

Eukaryotic cell expression vectors are well known in the art and

are available from several commercial sources. Typically, such

vectors provide convenient restriction sites for insertion of

25 the desired recombinant DNA molecule, and further contain

promoters for expression of the encoded genes which are capable

of expression in the eukaryotic cell, as discussed earlier.

Typical of such vectors are pSVO and pKSV-10 (Pharmacia), and

pPVV-1/PML2d (International Biotechnology, Inc.), and pTDT1

30 (ATCC, No. 31255).

In addition, in eukaryotic plasmids, one or more

transcription units are present that are expressed only in

eukaryotic cells. The eukaryotic transcription unit consists of

noncoding sequences and sequences encoding selectable markers.
The expression vectors of this invention also contain distinct
sequence elements that are required for accurate and efficient
polyadenylation. In addition, splicing signals for generating

5   mature mRNA are included in the vector. The eukaryotic plasmid
expression vectors can contain viral replicons, the presence of
which provides for the increase in the level of expression of
cloned genes. A preferred replication sequence is provided by
the simian virus 40 or SV40 papovavirus.

10   A preferrred expression system for producing the
recombinant molecules for use in this invention is an insect
system. In one such system, Autographa californica nuclear
polyhedrosis virus (AcNPV) is used as a vector to express
foreign genes. The virus grows in Spodoptera frugiperda (Sf9)

15   cells. The polypeptide-encoding nucleotide sequences of this
invention may be cloned into non-essential regions (in
Spodoptera frugiperda for example the polyhedrin gene) of the
virus and placed under control of an AcNPV promoter (for example
the polyhedrin promoter). Successful insertion of the

20   polypeptide-encoding nucleotide sequence will result in
inactivation of the polyhedrin gene and production of
non-occluded recombinant virus (i.e., virus lacking the
proteinaceous coat coded for by the polyhedrin gene). These
recombinant viruses are then used to infect cells in which the

25   inserted gene is expressed. See Smith, et al., J. Biol. Chem.,
46:584, (1983); Smith, U.S. Patent No. 4,215,051.

          In preferred embodiments, the host cell population is a
Drosophila cell culture that requires a compatible vector
including vectors functionally equivalent to those such as

30   p25-lacZ (see Bello and Couble, Nature, 346:480 (1990)) or
pRmHa-1, -2, or -3 (see Bunch, et al., Nucl. Acids Res.,
16:1043-1061 (1988)). In the preferred embodiment, the vector
is pRmHa-3, which is shown in Figure 1C. This vector includes a

metallothionein promoter, which is preferably upstream of the
site at which the MHC sequence is inserted, and the
polyadenylation site is preferably downstream of said MHC
sequence.   Insect cells and, in particular, _Drosophila_ cells are

5   preferred hosts according to the present invention. _Drosophila_
cells such as Schneider-2 (S2) cells, as further described in
Section D, have the necessary trans-acting factors required for
the activation of the promoter and are thus even more preferred.

The expression vector pRmHa-3 is based on the bacterial

10   plasmid pRmHa-1 (Figure 1A), the latter of which is based on
plasmid pUC18 and is deposited with the American Type Culture
Collection (ATCC, Rockville, MD), having the accession number
37253.   The pRmHa-3 vector contains the promoter, the 5'
untranslated leader sequence of the metallothionein gene with

15   the Eco RI and Stu I sites removed as shown in Figure 1C.   It
also contains the 3' portion of the _Drosophila_ ADH gene
including the polyadenylation site.   Therefore, cloned DNA is
transcriptionally regulated by the metallothionein promoter and
polyadenylated.   Construction of the pRmHa-1 plasmid is

20   described in Bunch, et al., _Nucl. Acids Res. 16_: 1043-1061
(1988).   Construction of the pRmHa-3 and pRmHa-2 plasmids (the
latter of which has a metallothionein promoter sequence that may
be removed as an Eco RI fragment) is described in the Examples.
With regard to pRmHa-3, a preferred plasmid for use according to

25   the present invention, Pst I, Sph I and Hind III are in the
promoter fragment and therefore are not unique. Xba I is in the
ADH fragment (4 bases from its 3' end) and is also not unique.
The following restriction sites are, however, unique in pRmHa-3
to facilitate cloning of the recombinant genes of this

30   invention: Eco RI, Sac I, Kpn I, Sma I, Bam HI, Sal I, Hinc 2,
and Acc I.

Mammalian cell systems that utilize recombinant viruses or
viral elements to direct expression may be engineered.   For

example, when using adenovirus expression vectors, the coding
sequence of a polypeptide may be ligated to an adenovirus
transcription/translation control complex, e.g., the late
promoter and tripartite leader sequence. This chimeric gene may
5   then be inserted into the adenovirus genome by _in vitro_ or _in
vivo_ recombination. Insertion in a non-essential region of the
viral genome (e.g., region E1 or E3) will result in a
recombinant virus that is viable and capable of expressing the
polypeptide in infected hosts (e.g., see Logan & Shenk, _Proc._
10 _Natl. Acad. Sci., USA_, 81:3655-3659, (1984)). Alternatively,
the vaccinia virus 7.5K promoter may be used. (e.g., see,
Mackett, et al., _Proc. Natl. Acad. Sci., USA_, 79:7415-7419,
(1982); Mackett, et al., _J. Virol._, 49:857-864, (1984);
Panicali, et al., _Proc. Natl. Acad. Sci., USA_, 79:4927-4931,
15 (1982)). Of particular interest are vectors based on bovine
papilloma virus which have the ability to replicate as
extrachromosomal elements (Sarver, et al., _Mol. Cell. Biol._,
1:486, (1981)). Shortly after entry of this DNA into mouse
cells, the plasmid replicates to about 100 to 200 copies per
20 cell. Transcription of the inserted cDNA does not require
integration of the plasmid into the host's chromosome, thereby
yielding a high level of expression. These vectors can be used
for stable expression by including a selectable marker in the
plasmid, such as the _neo_ gene. Alternatively, the retroviral
25 genome can be modified for use as a vector capable of
introducing and directing the expression of a polypeptide-
encoding nucleotide sequence of this invention in host cells
(Cone & Mulligan, _Proc. Natl. Acad. Sci., USA_, 81:6349-6353,
(1984)). High level expression may also be achieved using
30 inducible promoters, including, but not limited to, the
metallothionine IIA promoter and heat shock promoters.

For long-term, high-yield production of recombinant
proteins, stable expression is preferred. Rather than using

expression vectors which contain viral origins of replication, host cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter and enhancer sequences, transcription terminators, polyadenylation

5   sites, etc.), and a selectable marker. As mentioned above, the selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

10   For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et

15 al., Cell, 11:223, (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci., USA, 48:2026, (1962)), and adenine phosphoribosyltransferase (Lowy, et al., Cell, 22:817, (1980)) genes, which can be employed in tk⁻, hgprt⁻ or aprt⁻ cells

20 respectively. Also, antimetabolite resistance-conferring genes can be used as the basis of selection; for example, the genes for dhfr, which confers resistance to methotrexate (Wigler, et al., Proc. Natl. Acad. Sci., USA ,77:3567, (1980); O'Hare, et al., Proc. Natl. Acad. Sci., USA, 78:1527, (1981)); gpt, which

25 confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci., USA, 78:2072, (1981)); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., J. Mol. Biol., 150:1, (1981)); and hygro, which confers resistance to hygromycin (Santerre, et al., Gene, 30:147,

30 (1984)). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, Proc. Natl.

Acad. Sci., USA, 85:804, (1988)); and ODC (ornithine

decarboxylase) which confers resistance to the ornithine

decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO

(McConlogue L., In: Current Communications in Molecular Biology,

5   Cold Spring Harbor Laboratory ed., (1987).

Both prokaryotic and eukaryotic expression vectors are

familiar to one of ordinary skill in the art of vector

construction and are described by Ausebel, et al., In Current

Protocols in Molecular Biology, Wiley and Sons, New York (1993)

10   and by Sambrook et al., Molecular Cloning: A Laboratory Manual,

Cold Spring Harbor Laboratory, (1989).

For producing recombinant MHC class II α- and β-chains,

accessory molecules and antigen processing assisting molecules

for use in the compositions and methods of this invention, the

15   respective nucleotide regions are operatively inserted into an

expression vector of this invention as described herein. As

described in Section B, nucleic acids encoding the recombinant

polypeptides of this invention are obtained in a number of ways

one of which is by PCR amplification. One of the nucleotide

20   segments to be operatively linked to vector sequences encodes at

least a portion of MHC class II α- and β-chains. Preferably,

the respective nucleotide sequences for encoding the complete α-

and β-chains are separately inserted into an expression vector

for expression therefrom; however, it is also feasible to

25   construct a vector which also includes some non-coding MHC

sequences as well. The sequences for encoding accessory

molecules and antigen processing assisting molecules are

similarly inserted into separate expression vectors.

Alternatively, the invention contemplates the presence of

30   more than one polypeptide-encoding gene being present within the

same vector, the expression of which is driven by separate

regulatory elements, such as a promoter. In other words, the

nucleic acids for encoding both α- and β-chains may be

operatively ligated to the same expression vector with or
without one or more nucleic acid sequences encoding accessory
molecules. Thus, all possible combinations of expression vector
construction for producing the recombinant proteins of this
5   invention are contemplated.

        In addition to the complete encoding nucleotide sequences
as described above, soluble forms of the expressed recombinant
polypeptides of this invention are contemplated. The soluble
form differs from the non-soluble form in that it contains a
10   "stop" codon inserted prior to the transmembrane domain or other
functional location to generate soluble non-membrane anchorable
proteins.

D.   Synthetic Antigen Presenting Cells and Matrices for Peptide
15   Presentation

        1.   Synthetic Antigen Presenting Cells and Matrices

                In accordance with the present invention, the
recombinant MHC class II heterodimers and at least one accessory
molecule are operably linked to a matix comprising a support
20   such that the MHC class II and accessory molecules are present
in sufficient numbers to activate a population of CD4$^+$ T cells
lymphocytes when presented with a peptide complexed to the
extracellular portion of the MHC molecule. The peptide can be
bound to the MHC class II heterodimer before or after it is
25   linked to the support.

        The support can take on many different forms. It can be a
solid support such as a plastic or metal material, it can be a
porous material such as commonly used in separation columns, it
can be a liposome or red blood cell, or it can even be a cell or
30   cell fragment. As discussed in more detail below, in the case
where a cell serves as a support, the MHC class II and accessory
molecules can be produced by the cell for presentation on that

cell or for presentation on another support that can include a separate cell.

In the former situation, the MHC molecule is then linked to the cell by at least the transmembrane domain if not also the

5   cytoplasmic domain which would not be present in a soluble form of MHC class II. In the latter situation, the extracellular portions of MHC class II molecule and accessory molecule can be linked to a support by providing an epitope which reacts to an antibody immobilized on the support. In addition, the MHC or

10 assisting molecules can be produced with or linked to (His)$_6$ which would react with nickel in forming part of the support. Other means to immobilize or link MHC molecules to a support are well known in the art.

As discussed above, the support can be a cell membrane or

15 an entire cell. In such a case, an eukaryotic cell line is modified to become a synthetic antigen presenting cell line for use in presenting peptide in the context of MHC class II to T cell lymphocytes. Because empty MHC molecules are thermolabile, it is preferred that the cell culture be poikilotherm and

20 various cell lines are discussed in detail below.

A preferred cell line of the present invention is capable of continuous growth in culture and capable of expressing mammalian MHC class II molecules and accessory molecules on the cell surface. Any of a variety of transformed and

25 non-transformed cells or cell lines are appropriate for this purpose, including bacterial, yeast, insect, and mammalian cell lines. (See, e.g., _Current Protocols in Molecular Biology_, John Wiley & Sons, NY (1991), for summaries and procedures for culturing and using a variety of cell lines, e.g., _E. coli_ and

30 _Sarcomyces cerevisiae_).

Preferably, the cell line is a eukaryotic cell line. More preferably, the cell line is poikilothermic (i.e., less sensitive to temperature challenge than mammalian cell lines).

More preferably, it is an insect cell line. Various insect cell
lines are available for use according to the present invention,
including moth (ATCC CCL 80), armyworm (ATCC CRL 1711), mosquito
larvae (ATCC lines CCL 125, CCL 126, CRL 1660, CRL 1591, CRL

5   6585, CRL 6586), silkworm (ATCC CRL 8851) and butterfly
(Spodoptera frugiperda (Sf9 cells, ATCC CRL 1711). In a
preferred embodiment, the cell line is a Drosophila cell line
such as a Schneider cell line (see Schneider, J. Embryol. Exp.
Morph., 27:353-365 (1972)); preferably, the cell line is a

10   Schneider 2 (S2) cell line (S2/M3) adapted for growth in M3
medium (see Lindquist, et al., Drosophila Information Service,
58:163 (1982)). Schneider 2 (S2) cells have been deposited
pursuant to Budapest Treaty requirements with the American Type
Culture Collection (ATCC), Rockville, MD, on February 18, 1992,

15   and was assigned accession number CRL 10974.

          To generate a synthetic antigen presenting cell of this
invention, one or more expression vectors for directing the
expression of a selected MHC class II heterodimer in conjunction
with one or more accessory molecules is introduced into a

20   recipient host cell. In addition, in alternative embodiments,
vectors for expressing antigen processing assisting molecules
including HLA-DM and invariant chain are also introduced into
the recipient cells. The genes for the above have been
described in Sections B and C.

25   Thus, in order to prepare synthetic antigen presenting
cells or matrices, the expression vectors for encoding
recombinant polypeptides of this invention are transfected, i.e,
introduced, into a selected host cell. The selection of
expression vectors as well as the construction thereof is

30   dependent upon the desired outcome for $CD4^+$ activation as
previously discussed as as reiterated below. Transfection, also
referred to as transformation, may be accomplished via numerous
methods, including the calcium phosphate method, the

DEAE-dextran method, the stable transfer method, electroporation, or via the liposome mediation method. Numerous texts are available which set forth known transfection methods and other procedures for introducing nucleotides into cells;

5   see, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, NY (1991). Following introduction of one or more vectors, the recipient cell is said to be transformed, the selection of which can either be transient or stable.

A culture of cells is first established. A cell line is

10   chosen for transfection because it lacks at least one of the genes being introduced. It has been found that insect cells are advantageous not only because they are poikilothermic, but because they lack these genes and the mechanisms which would otherwise produce MHC molecules bound to peptides. This allows

15   for greater control over the production of peptide-bound MHC molecules, and the production of empty MHC molecules.

The selected cells are then transformed by the introduction of an expression vector containing an expressible MHC class II α-chain gene operably linked to a first promoter and an

20   expressible MHC class II β-chain gene operably linked to a secong promoter. A first expressible accessory molecule gene operably linked to a third promoter in a vector is also introduced into the above cell. In a further embodiment, an expressible antigen processing assisting gene operably linked to

25   a fourth promoter in a vector is introduced into the above cell.

In a more preferred embodiment, the vector comprises Drosophila expression plasmid pRmHa-3, described in Section C, into which expressible nucleotide sequences encoding the above

30   recombinant proteins have been inserted using techniques disclosed herein. Preferably, the nucleotide sequences encoding the MHC class II chains, those encoding at least one accessory molecule and those encoding antigen processing assisting

molecules are operably linked to separate expression plasmids that are individually cotransfected into the cultured cells. Alternatively, the nucleotide sequences may be operably linked to separate promoters in the same expression plasmid and

5   cotransfected via that same plasmid. The MHC class II α- and β-chains are preferably from a different species, more preferably, a homeotherm such as mammals and, optimally, humans.

It is preferred that at least one of the genes and, in particular, the MHC class II chain genes be linked to an

10   inducible promoter. This allows control over the production of MHC molecules so that they are only produced at a time when the peptide of interest is available either internally or externally and presented in the culture to react with the produced MHC molecules. This minimizes undesirable MHC molecule/peptide

15   complexes.

Thus, the preferred cell line is a poikilotherm cell line that has separate vectors each containing a MHC class II α- and β-chain gene respectively operably linked to a first and second promoter. Preferably, the promoters are inducible to control

20   the expression of the MHC class II chains. In addition, the cell contains a third vector containing at least a first expessible accessory molecule gene operably lined to a third promoter. In a further embodiment, the cell also contains a fourth vector containing an expressible antigen processing

25   assisting gene operably linked to a fourth promoter. It is preferred that the cell assembles empty MHC molecules and presents them on the cell surface so that the peptides specific for a particular MHC class II haplotype or for a variant allele can be selected as desired.

30   The selection of compatible MHC class II α- and β-chain genes for use in conjunction with one or more particular accessory molecule-encoding genes is dependent upon the T cell activation profile desired. For example, as described in the

Examples, recombinant B7.1 or B7.2 alone or together in
conjunction with recombinant murine IA^d MHC class II expressed on
the surface of <u>Drosophila</u> APC resulted in proliferation of CD4$^+$ T
cells having a Th2 profile of increased production of IL-4 and

5   IL-10. In contrast, when either B7.1 or B7.2 were expressed on
the surface of <u>Drosophila</u> APC with ICAM-1 along with the same
MHC molecules, the activation of CD4+ T cells resulted in a Th1
profile with increased IL-2 production and decreased IL-4 and
IL-10 production.

10   Thus, the invention contemplates the production of
synthetic APC having on the cell surface any combination of a
MHC class II haplotype heterodimer with any one of the accessory
molecules of this invention. Particularly preferred
combinations include MHC class II with either a costimulatory

15   molecule including B7.1 or B7.2, an adhesion molecule including
ICAM-1, ICAM-2, ICAM-3 or LFA-3, or a survival molecule
including Fas ligand (FasL). As described above, more than one
of each category of accessory molecules can be co-expressed, as
for example, B7.1 and B7.2. Alternative preferred embodiments

20   include the permutations where two accessory molecules of
different categories are co-expressed on the cell surface. In
other words, an adhesion molecule with a costimulatory molecule,
and adhesion molecule with a survival molecule, a costimulatory
molecule with a survivial molecule. In a further embodiment,

25   three accessory molecules of the different categories described
herein are co-expressed on the APC surface. It is also
contemplated that in all of these embodiments, more than one
member of a particular category may be expressed in conjunction
with more than one member of other categories. The particular

30   selected combinations are effective on the surface of the cells
from which they are expressed or when anchored on the surface of
a matrix of this invention. The actual combinations prepared
are selected on the basis of T cell activation outcome in view

of the expressed MHC class II molecules complexed with antigenic peptide. Thus, depending on the complex of MHC class II/peptide, the T cell activation outcome may be distinct despite having the same expressed accessory molecules.

5   In a further embodiment, antigen processing assisting genes are co-transfected with any of the above-described combinations to provide for enhanced internal peptide processing and loading. This aspect thus does not involve the generation of empty MHC class II molecules on the cell surface for subsequent peptide

10   complexation. Rather, the expression of invariant chain, HLA-DM or lysosomal enzymes is utilized to allow for optimal processing and loading of proteolytic peptide fragments following cell internalization. The APC of this invention thus can function in either motif of having the recombinant MHC class II heterodimers

15   being loaded either intracellularly or extracellularly.

       Successfully transformed cells, i.e., cells that contain at least one expression vector capable of directing the expression of nucleotide sequences according to the present invention, can be identified via well-known techniques. For example, cells

20   resulting from the introduction of a cDNA or rDNA of the present invention can be cloned to produce individual colonies. Cells from those colonies can be harvested, lysed, and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, J. Mol. Biol. 98: 503

25   (1975). In addition to directly assaying for the presence of rDNA, successful transformation or transfection may be confirmed by well-known immunological methods when the rDNA is capable of directing the expression of a subject MHC class II protein of accessory molecule. For example, cells successfully transformed

30   with one or more expression vectors may produce proteins displaying particular antigenic properties which are easily determined using the appropriate antibodies, such as anti-class II of particular haplotypes. In addition, successful

transformation/transfection may be ascertained via the use of an additional vector bearing a marker sequence, such as neomycin resistance, as described hereinabove.

It is also preferable that the culture be stable and capable of sustained growth at reduced temperatures. For example, it is preferred that the culture be maintained at about room temperature, e.g., about 24-27°C. In other embodiments, the culture is maintained at higher temperatures, particularly during the process of activating CD4⁺ cells. It is thus preferred that a culture according to the present invention be capable of withstanding a temperature challenge of about 30°C to about 37°C.

In order to prepare the culture for expression of empty or MHC class II molecules in conjunction with a least one accessory molecule of this invention and optionally antigen processing assisting molecules, the culture may first require stimulation, e.g., via $CuSO_4$ induction, for a predetermined period of time. After a suitable induction period, e.g., about 12-48 hours, peptides may be added at a predetermined concentration (e.g., about 0.2 µg/ml to 20 µg/ml). Proteins and peptides for both internal and external loading are prepared as discussed below. After a further incubation period, e.g., for about 12 hours at 27°C, the culture is ready for use in the activation of CD4⁺ cells. While this additional incubation period may be shortened or perhaps omitted, the culture tends to become increasingly stable to temperature challenge if it is allowed to incubate for a time prior to addition of resting or naive CD4⁺ cells. For example, cultures according to the present invention to which peptide has been added are capable of expressing significant amounts of peptide-loaded MHC class II molecules even when incubated for extended periods of time at 37°C.

Nutrient media useful in the culturing of transformed host cells are well known in the art and can be obtained from

numerous commercial sources. In embodiments wherein the host cell is mammalian, a "serum-free" medium is preferably used.

The resulting recombinant expressed MHC class II molecules bind to a particular peptide and are present in sufficient

5   numbers with at least one accessory molecule on the surface of the APC to activate a population of T cell lymphocytes against the MHC class II/peptide complex.

Where the cell line already produces one or more of the desired molecules, it is only necessary to transfect the culture

10   with an expressible gene for the gene which is lacking in the cells. For example, if the cells already present the MHC molecules on their surface, it is only necessary to transfect the culture with a vector containing an expressible gene for the accessory molecule.

15   As previously discussed, a protein or peptide can be introduced into the cell culture at the time the cells are producing MHC class II molecules for internal processing. Through methods such as osmotic shock, the peptides can be introduced in the cell and bind to the produced MHC molecules.

20   Alternatively, particularly in the case poikilotherm cell lines, the MHC molecules will be presented empty on the cell surface. The peptide can then be added to the culture and bound to the MHC molecules as desired. For simplicity, while one peptide is described herein, the methods of this invention contemplate the

25   screening of peptide libraries for identifying novel antigenic peptides for use in the therapeutic methods of this invention.
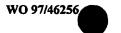
After the cells are produced having a MHC class II heterodimer and at-least one accessory molecules on the cell surface, the cells can be lyophilized to generate cell fragments

30   for use in activating a population of CD4$^+$ T cell lymphocytes.

Transfected cultures of cells are also used to produce extracellular portions of MHC class II molecules and accessory molecules. The use of extracellular portions in conjunction

with supports such as solid supports has certain advantages of
production. Where living cells are used to provide a synthetic
antigen presenting cell, at least three genes, two to produce
the MHC class II heterodimer and one for the accessory molecule

5   must be introduced to the cell. Often, additional genes such as
for antibiotic resistance are also transfected.

Where a solid support system is being used, one cell line
is used to produce the extracellular portions of MHC class II
molecules while another cell line is used to produce the

10   extracellular portion of an accessory molecule. The MHC
molecule portions and the accessory molecule portions are then
harvested from their respective cultures. The molecules are
then linked to an appropriate support in sufficient numbers to
activate a population of T cells. From a production standpoint,

15   two different cultures can be used, but it is also possible to
use the same culture, however, requiring that the culture be
transfected with the additional gene for expressing the
extracellular portion of an accessory molecule.

A further modification of this embodiment is to provide a

20   third culture of cells which is transfected with an expressible
second accessory molecule gene. For example, the second culture
of cells produces extracellular portions of the costimulatory
molecule while the third culture of cells produce an
extracellular portion of an adhesion molecule. The adhesion

25   molecule portions are harvested and linked to the support.
In preparing the extracellular portions of a MHC class II
heterodimer to be linked to a support, soluble molecules are
prepared as previously discussed. These molecules generally
lack the transmembrane and cytoplasmic domain in the MHC

30   molecule.

2. Peptides

Virtually all cellular proteins in addition to viral antigens are capable of being used to generate relevant peptide fragments that serve as potential MHC class II-specific

5 peptides. The methods and compositions of this invention provide for MHC class II molecules that have an increased capacity to specifically activate CD4⁺ cells.

The peptides of the present invention bind to MHC class II molecules. The binding occurs under biological conditions which

10 can be created in vivo as well as in vitro. The exact nature of the binding of the peptides need not be known for practice of the invention.

Peptides that bind MHC class II molecules are variable in length and their anchor residues lie at various distances from

15 the ends of the peptide. In one aspect, the peptides prepared for loading onto the MHC molecules are of a single species; i.e., that all peptides loaded onto the MHC be identical in size and sequence so as to produce monoantigenic peptide-loaded MHC class II molecules. In alternative embodiments, peptides are

20 heterogenous and may comprise a random library of peptides to allow for selection of unique members that result in desired T cell activation profiles as previously described. The production and screening of random synthetic peptide libraries is familiar to one of ordinary skill in the art and is described

25 in U.S. Patent Nos. 5,556,762, 5,510,240, 5,498,530, 5,432,018, 5,382,513, 5,338,665 and 5,270,170, the disclosures of which are hereby incorporated by reference.

Peptides may be presented to the cells via various means. Preferably, peptides are presented in a manner which allows them

30 to enter an intracellular pool of peptides. For example, peptides may be presented via osmotic loading. Typically, peptides are added to the culture medium. The peptides may be added to the culture in the form of an intact polypeptide or

protein which is subsequently degraded via cellular processes,
e.g., via enzymatic degradation. Alternatively, the intact
polypeptide or protein may be degraded via some other means such
as chemical digestion (e.g. cyanogen bromide) or proteases (e.g.

5 chymotrypsin) prior to its addition to the cell culture. In
other embodiments, the peptides are presented in smaller
segments which may or may not comprise antigenic amino acid
sequences in conjunction with a particular MHC class II
haplotype.

10   Preferably, a sufficient amount of protein(s) or peptide(s)
is added to the cell culture or synthetic matrix to allow the
MHC class II molecules to bind and subsequently present a large
density of the peptide. preferably, with the same kind of
peptide attached to each MHC heterodimer, on the surface of the

15 synthetic APC or matrices of this invention.

In another embodiment of the invention, peptides are added
to transfected cells of the present invention in order to
enhance the thermostability of the MHC molecules expressed by
the cells. As noted above, peptides are preferably added to the

20 culture medium. Antigenic peptides that bind to the MHC class
II molecules serve to thermostabilize the MHC molecules and also
increase the cell surface expression. Cultures with added
peptides which bind to the MHC molecules are thus significantly
less susceptible to temperature challenge than cultures without

25 added peptide.

E.   Methods of Altering CD4+ T Cell Responses
     1.   Th1 and Th2 CD4+ T Cell-Mediated Diseases
          Inducing a naive T cell into a desired activated T

30 cell type or deviating the effector function of an activated T
cell from a Th1 type to a Th2 type and visa versa is one of the
aims of the present invention, especially with regard to

therapeutic methods in treating various CD4⁺ T cell-mediated disease conditions.

The differentiation of a proliferating CD4⁺ T cell into either an inflammatory T cell or a helper T cell is dependent on

5   the cytokines produced by infectious agents, principally IL-12 and IL-4, the influence of accessory molecules and on the nature of the MHC class II/peptide complex. As previously discussed, cell-mediated immunity involves the destruction of intracellular pathogens by macrophages activated by Th1 inflammatory cells

10 directed primarily to intracellular parasites including such parasites as Mycobacterium, Leishmania, Pneumocystis and the like. In contrast, humoral immunity depends on the production of antibody by B cells activated by helper T cells directed primarily at extracellular pathogens including Clostridium,

15 Staphylococcus, Streptococcus, Polio virus, Pneumocystis and the like.

For example, recovery from certain types of infections, such as Leishmania, is associated with preferential production of IL-2/IFN-γ. Mice that mount a Th2 response to Leishmania

20 fail to contain the infection and ultimately die. Inappropriate production of cytokines of the Th2 type response has been frequently linked to allergic type diseases such as asthma and contact sensitivity.

Perhaps the strongest association of human disease with

25 skewed patterns of cytokine production is the association of Th1 responses and Th1 type cytokines with autoimmune disease. Strong evidence in experimental models indicates that many types of autoimmunity including diabetes, experimental models for multiple sclerosis, autoimmune thyroiditis, and the like are

30 mediated by Th1 type CD4⁺ T cells. The expression of Th2-associated cytokines, such as IL-4, in these models interfere with the development of autoimmune disease. Th2 type cytokines

dampen the response of Th1 type cells while the Th1 type
cytokines antagonize the development of Th2 type responses.

In view of the association of particular activated T cell
subsets with particular disease conditions, a need therefore
5   exists to be able to direct the proliferation and activation of
CD4$^+$ T cells to a desired T cell subset, a process that is
extremely beneficial in altering the course of disease. One
potential solution is to activate _in vitro_ CD4$^+$ T cells that are
first isolated from a subject who may optionally be having
10   either allergy or autoimmune conditions to produce cells
secreting a preferred cytokine profile. The resultant activated
T cells are then reintroduced to the subject to alter the course
of disease and perhaps even provide a long term cure.

Alternative embodiments are directed at the ability to
15   "vaccinate" a potentially responsive individual against the
development of either a Th1 or Th2 response, whichever is
applicable to that individual. In other words, the selective
induction of a particular T cell subset may be achieved by
inhibiting naive cells from developing toward the undersired
20   phenotype. For example, in a potentially atopic individual,
preventing a deleterious Th2 response would be beneficial as
described by Hetzel and Lamb, _Clinical Immunol. Immunopath._,
73:1-10 (1994).

In still a further embodiment, the compositions and methods
25   of this invention are useful for actively stimulating the
development of naive T cells toward the desired phenotype.
Existing therapeutic models to date include the use of anti-
cytokine antibodies are carrier proteins, the use of
idiotype/GM-CSF fusion protein vaccines to prolong effects of
30   exogenous cytokines, use of selected adjuvants, use of liposome-
encapsulated allergens, use of peptide analogs and the like as
reviewed by Hetzel and Lamb, _id._ The authors, however, do state
that for chronic Th2 responses to allergens _in vivo_, little

experimental data exists for the possibility of effecting a desired downregulation of the Th2 response.

In view of the foregoing, the compositions and methods of this invention provide a valuable means to accomplish the

5 therapeutic interventions discussed above.

The present invention allows one to define activation conditions that reproducibly generate CD4$^+$ T cell subsets that produce the desired therapeutic cytokine profile. Expression of particular cytokines is linked to a particular antigen presenting cell

10 (APC) and their associated accessory molecules. Since both the cytokines produced by the APC and the coordinately expressed accessory molecules are themselves regulated by multiple factors, including the type of antigen, the affinity of the T cell receptor (TCR)-antigen interaction, antigen concentration

15 and the like, predicting the outcome of T cell activation upon antigen presentation is historically very difficult. Indeed, as additional accessory molecules have been proposed for the activation process _in vivo_, it has become increasingly clear that many diverse molecules are involved in the regulation of T

20 cell responses and act in combinatorial fashion to effect the outcome of T cell activation.

The present invention provides the generation of synthetic APC that present, in a neutral background, MHC class II molecules in combination with defined accessory molecules that

25 are expressed preferably in a nonmammalian insect cell. The advantage of using the insect cells as the expression and presentation vehicles for the MHC class II/accessory molecule compositions of this invention is that the cells do not endogenously produce regulatory cytokines and do not express

30 mammalian accessory molecules. This overcomes the inherent unpredictability of using mammalian APC that express many molecules that are capable of altering the T cell response. The present invention thus provides the ability to isolate

individual presenting molecules and accessory molecules for expression in selected combinations that permits reproducibility and predictability not available in other approaches.

5   2.   Therapeutic Methods

As discussed above, the present invention relates to a method for activating CD4⁺ T cells into differentiated armed effector T cell subtypes. The method relates to providing a synthetic APC or matrix having anchored on the external surface
10   a recombinant MHC class II heterodimer that is capable of binding a peptide. The compositions also have at least one accessory molecule presented on the cell surface. Naive or activated CD4⁺ T cells can be obtained by removal from an individual to be treated. The antigen presenting cells are then
15   contacted with the CD4⁺ T cells for a sufficient period of time to activate the T cells into proliferating and differentiating into a desired T cell phenotype.

The activated CD4⁺ T-cells are separated from the cell line and put into a suspension in an acceptable carrier and
20   administered to the individual.

It is preferred that human genes are used and, therefore, human molecule analogs are produced. As shown in prior U.S. Patent No. 5,314,813, murine systems provide particularly useful models for testing the operation of T cell activation and
25   demonstrate the applicability of the process for human systems. See also Sykulev et al., Immunity, 1:15-22 (1994).

a.   Isolation of Resting or Activated CD4⁺ T Cells

Resting (or naive) as well as activated CD4⁺ cells
30   that have not been activated to target a specific antigen presented in the context of MHC class II are extracted from an individual for incubation or exposure to the transformed cultures of the present invention. Naive cells can be

distinguished from primer cells primarily based on the cell surface markers CD455RA and CD45.

It is also preferred that CD4$^+$ cells are obtained from an individual prior to the initiation of other treatment or therapy
5   which may interfere with the CD4$^+$ cells' ability to be specifically activated. For example, if one is intending to treat an individual with an autoimmune disease, it is preferable to obtain a sample of cells and culture prior to the initiation of adjunctive therapy such as steriod treatment or during a
10   window of time when the patient is not being treated at all.

When activating CD4$^+$ T cells to alter the T cell-mediated immune response in a patient, the patient is first analyzed for a patient-specific profile to assess the T cell phenotypic disease state for instituting appropriate counter therapy
15   involving production of the opposing T cell phenotype and cytokines. Cytokine profiles are established with anti-cytokine antibodies that are available from ATCC and by methods described in U.S. Patent Nos. 5,405,751, 5,322,787 and 5,209,920, the disclosures of which are hereby incorporated by reference.
20   Preferred cytokine analyses include interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor (TNF), interleukin-4 (IL-4), interleukin-10 (IL-10) and the like. As previously discussed, particular cytokine profiles are associated with T cell phenotypes and disease states.

25   In particular, where the condition is an autoimmune disease including multiple sclerosis, autoimmune thyroiditis, systemic lupus erythromatosus, myasthenia gravis, Crohn's disease and inflammatory bowel disease, the cytokine profile is produced by a Th1 type response characterized by increased IL-2, IFN-γ and
30   TNF. In contrast, where the condition is an allergy, such as asthma and contact sensitivity, the cytokine profile is produced by a Th2 type response characterized by increased IL-4 and IL-10.

After analyzing the patient cytokine profile and disease
state, patient-isolated CD4⁺ T cells are contacted _in vitro_ with
the synthetic APC, cell fragments or matrices of this invention
as described below in a sufficient amount for a sufficient time
5　to induce the contacted cells to proliferate and differentiate
into activated CD4⁺ T cells that produce a functionally opposing
cytokine profile. That is, if the patient were characterized as
being a Th1 type responder, the antigen to be presented to the
patients CD4⁺ T cells would be that necessary to induce the cells
10　to proliferate and differentiate into a Th2 type. The opposite
treatment modality is performed if the patient is characterized
with a Th2 type response. Thus, once the opposing activated
cells are returned to the patient as described below, the
therapeutic goal of effecting an alteration in T cell phenotype
15　response is attained.

Methods of extracting and culturing lymphocytes are well
known. For example, U.S. Patent No. 4,690,915 to Rosenberg
describes a method of obtaining large numbers of lymphocytes via
lymphocytopheresis. Appropriate culturing conditions used are
20　for mammalian cells, which are typically carried out at 37°C.

Various methods are also available for separating out
and/or enriching cultures of CD4⁺ cells. Some examples of
general methods for cell separation include indirect binding of
cells to specifically-coated surfaces. In another example,
25　human peripheral blood lymphocytes (PBL), which include CD4⁺
cells, are isolated by Ficoll-Hypaque gradient centrifugation
(Pharmacia, Piscataway, NJ). PBL lymphoblasts may be used
immediately thereafter or may be stored in liquid nitrogen after
freezing in FBS containing 10% DMSO (Sigma Chemical Co., St.
30　Louis, MO), which conserves cell viability and lymphocyte
functions.

Alternative methods of separating out and/or enriching
cultures of precursor cells include both positive and negative

selection procedures. For positive selection, after
lymphocyte-enriched PBL populations are prepared from whole
blood, subpopulations of CD4⁺ lymphocytes are isolated therefrom
by affinity-based separation techniques directed at the presence

5   of the CD4 co-receptor antigen. These affinity-based techniques
fluorescence-activated cell sorting (FACS), cell adhesion,
magnetic bead separation and like methods. (See, e.g., Scher
and Mage, in Fundamental Immunology, W.E. Paul, ed., pp.
767-780, River Press, NY (1984).) Affinity methods may utilize

10   anti-CD4 co-receptor antibodies as the source of affinity
reagent. Alternatively, the natural ligand, or ligand analogs,
of CD4 receptor may be used as the affinity reagent. Various
anti-T cell and anti-CD4 monoclonal antibodies for use in these
methods are generally available from a variety of commercial

15   sources, including the American Type Culture Collection
(Rockville, MD) and Pharmingen (San Diego, CA).

Negative selection procedures are utilized to effect the
removal of non-CD4 from the CD4+ population. This technique
results in the enrichment of CD4⁺ cells from the T and B cell

20   population of leucophoresed patients. Depending upon the
antigen designation, different antibodies may be appropriate.
For example, monoclonal antibodies OKT4 (anti-CD4, ATCC No. CRL
8002) OKT 5 (ATCC Nos. CRL 8013 and 8016), OKT 8 (anti-CD8, ATCC
No. CRL 8014), and OKT 9 (ATCC No. CRL 8021) are identified in

25   the ATCC Catalogue of Cell Lines and Hybridomas (ATCC,
Rockville, MD) as being reactive with human T lymphocytes, human
T cell subsets, and activated T cells, respectively. Various
other antibodies are also available for identifying and
isolating T cell species, including precursors and naive and

30   activated memory mature peripheral T cells.

b.   <u>In Vitro Activation of CD4⁺ Cells</u>

In order to optimize the <u>in vitro</u> conditions for
the generation of specific CD4⁺ T cell phenotypes, the culture of
antigen presenting cells is maintained in an appropriate medium.

5   Preferably, when using a support of this invention that is an
intact cell, the antigen-presenting cells are <u>Drosophila</u> cells,
which are preferably maintained in serum-free medium (e.g.
Excell 400). In alternative embodiments, however, when the
support is a cell fragment or a matrix of an artificial support

10   as previously described, the culture medium is selected to
maintain the viability of the target cells.

Prior to incubation of the synthetic APC, cell fragments or
matrices of this invention with the T cells to be activated, an
amount of antigenic peptide is provided to the APC or matrices

15   in sufficient quantity to become loaded onto the human MHC class
II molecules for expression on the surface of the APC or
matrices. As previously discussed, peptide loading can occur
intracellularly or extracellularly. Both aspects are
accordingly encompassed in the activation process described

20   herein but for simplicity, loading of peptides is generically
described as the details of peptide presentation to MHC class II
heterodimers and loading have been previously discussed.
Moreover, individual peptides as well as peptide libraries are
contemplated for use in preparing activated CD4⁺ T cells also as

25   previously described. According to the present invention, a
sufficient amount of peptide is an amount that will allow about
200 to about 500,000 and preferably about 200 to 1,000 or more,
MHC class II molecules loaded with peptide to be expressed on
the surface of each synthetic APC or matrix. Preferably, the

30   above compositions are incubated with 0.2 μg/ml up to 20 μg/ml
peptide.

The isolated CD4⁺ cells are then incubated in culture with
the appropriate peptide-loaded MHC class II heterodimers

expressed on synthetic APCs or matrices for a time period
sufficient to activate CD4⁺ cells. Preferably, the CD4⁺ cells
shall thus be activated in an antigen-specific manner. The
ratio of CD4⁺ cells to antigen-presenting cells may vary from

5   individual to individual and may further depend upon variables
such as the amenability of an individual's lymphocytes to
culturing conditions and the nature and severity of the disease
condition or other condition for which the within-described
treatment modality is used. Preferably, however, the

10 lymphocyte:antigen-presenting cell or matrix ratio is preferably
in the range of about 1:1 to 300:1.

The effector/antigen-presenting culture may be maintained
for as long a time as is necessary to activate and enrich for a
population of a therapeutically useable or effective number of

15 CD4⁺ cells. In general terms, the optimum time is between about
one and five days, with a maximum specific level generally being
observed after three to five days of culture. In one embodiment
of the present invention, _in vitro_ activation of CD4⁺ cells is
detected within a brief period of time after transfection of a

20 cell line.

Preferably, the activation of CD4⁺ cells is optimal within
one week of exposure to antigen-presenting cells. Thereafter,
in a preferred embodiment, the activated CD4⁺ cells are further
purified by isolation procedures including density gradients,

25 rosetting with antibody-red blood cell preparations, column
chromatography and the like. Following the purification, the
resulting CD4⁺ cell preparation is further expanded by
maintenance in culture for a period of time to obtain a
population of 10^9 activated CD4⁺ cells. This period may vary

30 depending on the replication time of the cells but may generally
be 14 days.

c.   <u>Separation of CD4⁺ Cells from Synthetic APC or
      Matrices</u>

Activated CD4⁺ cells may be effectively separated
from the antigen presenting compositions of this invention using
one of a variety of known methods. For example, monoclonal
antibodies specific for the MHC class II heterodimers, for the
peptides loaded thereon, or for the CD4⁺ cells (or a segment
thereof) may be utilized to bind their appropriate complementary
ligand. Antibody-tagged cells may then be extracted from the
stimulator-effector cell admixture via appropriate means such as
via well-known flow cytometric or magnetic bead separation
methods. Density gradients can also be used to separate blast
cells.

d.   <u>Therapeutic Treatment with Activated CD4⁺ Cells</u>

Effective amounts of the activated CD4⁺ cells can
vary between <u>in vitro</u> and <u>in vivo</u> uses, as well as with the
amount and type of target cells expressing the antigenic peptide
used in activating the CD4⁺ population. The amount will also
vary depending on the condition of the patient and should be
determined via consideration of all appropriate factors by the
practitioner. Preferably, however, about 1 X 10⁶ to about 1 X
10¹², more preferably about 1 X 10⁸ to about 1 X 10¹¹, and even
more preferably, about 1 X 10⁹ to about 1 X 10¹⁰ activated CD4⁺
cells are utilized for adult humans, compared to about 5 X 10⁶ -
5 X 10⁷ cells used in mice.

Preferably, as discussed above, the activated CD4⁺ cells are
harvested from the synthetic APC or matrix in culture prior to
administration of the CDr⁺ cells to the individual being treated.
It is important to note, however, that unlike other present and
proposed treatment modalities, in a preferred embodiment, the
present method uses a cell culture system of <u>Drosophila</u> APC or
acellular matrices that are not tumorigenic. Therefore, if

complete separation of cells and activated CD4⁺ cells is not
achieved, there is no inherent danger known to be associated
with the administration of a small number of synthetic APC or
matrices, whereas administration of mammalian tumor-promoting

5   cells may be extremely hazardous.

Methods of re-introducing cellular components are known in
the art and include procedures such as those exemplified in U.S.
Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No.
4,690,915 to Rosenberg. For example, administration of

10 activated CD4⁺ cells via intravenous infusion is appropriate.

Examples

The following examples are intended to illustrate, but not
limit, the present invention.

15

1.   Preparation of pRmHa-3 Expression Vector

The pRmHa-3 expression vector for use in expressing MHC
proteins in Drosophila Schneider 2 (S2) cells as described in

20 this invention was constructed by ligating a Sph I-linearized
pRmHa-1 DNA expression vector with a DNA fragment resulting from
a Sph I restriction digest of a pRmHa-2 expression vector to
form the pRmHa-3 expression vector as described below. The
ligating of the linearized pRmHa-1 with the pRmHa-2 fragment in

25 this manner was performed to remove one of two Eco RI
restriction endonuclease cloning sites present in pRmHa-1.
Thus, the resultant pRmHa-3 expression vector contained only one
Eco RI restriction site in the multiple cloning site
(polylinker) into which various MHC class II-encoding DNA

30 fragments were inserted as described in the Examples.

A.   Preparation of pRmHa-1 Expression Vector

The pRmHa-1 expression vector, containing a
metallothionein promoter, metal response consensus sequences
(designated MT) and an alcohol dehydrogenase (ADH) gene

5 containing a polyadenylation signal isolated from Drosophila
melanogaster, was constructed as described by Bunch et al.,
Nucl. Acids Res.,16:1043-61 (1988). The plasmid expression
vector, pUC18, having the ATCC accession number 37253, was used
as the source vector from which subsequent vectors described

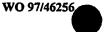10 herein were derived. The pUC18 plasmid contains the following
restriction sites from 5' to 3' in the multiple cloning site,
all of which are not illustrated in the schematic
representations of the pUC18-derived vectors in Figure 1: Eco
RI; Sac I; Kpn I; Sma I and Sma I located at the same position;

15 Bam HI; Xba I; Sal I, Acc I and Hinc II located at the same
position; Pst I; Sph I and Hind III. The pUC18 vector was first
digested with Hind III to form a linearized pUC18. Blunt ends
were then created by filling in the Hind III ends with DNA
polymerase I large fragment as described by Maniatis et al.,

20 Molecular Cloning: A Laboratory Manual, eds. Cold Spring
Harbor Laboratory, New York (1982).

The resultant linearized blunt-ended pUC18 vector was
ligated with a 740 base pair (bp) Hinf I fragment from the
Drosophila melanogaster ADH gene containing a polyadenylation

25 signal. The ligated ADH allele was first isolated from the
plasmid pSACI, described by Goldberg et al., Proc. Natl. Acad.
Sci., USA, 77:5794-5798 (1980), by digestion with Hinf I
followed by blunt ending with Klenow resulting in the nucleotide
sequence listed in SEQ ID NO 1. The pSACI vector containing the

30 ADH allele was constructed by subcloning into pBR322 (ATCC
accession number 31344) a 4.7 kilobase (kb) Eco RI fragment of
Drosophila DNA selected from a bacteriophage lambda library
containing random, high molecular weight (greater than 15 kb).

The 5' Hinf I restriction site occurred naturally in the ADH gene at position 1770 as described by Kreitman, _Nature_, 304:412-417 (1983). The 3' Hinf I site was derived from the pUC18 vector into which the ADH gene had been cloned. This

5 position was four bases 3' to the Xba I site at position 2500 of the ADH gene. The ADH segment extended from 35 bp upstream of the polyadenylation/cleavage sequence in the 3' untranslated portion of the ADH mRNA to 700 bp downstream of the polyadenylation signal. The resultant pUC18-derived vector

10 containing the ADH gene fragment was designated pHa-1 as shown in Figure 1A.

A 421 bp Eco RI/Stu I MT gene fragment for insertion into pHa-1 was obtained from a clone containing DNA of approximately 15.3 kb in a _Drosophila melanogaster_ genomic DNA library. The

15 library, prepared with a Mbo I partial digestion of imaginal DNA, was cloned in the lambda derivative EMBL4. The 421 bp fragment contained the MT promoter and metal response consensus elements of the _Drosophila_ MT gene (Maroni et al., _Genetics_, 112:493-504 (1986)). This region, containing the promoter and

20 transcription start site at nucleotide position +1, corresponded to position -370 to nucleotide position +54 of the MT gene (SEQ ID NO 2). The resultant fragment was then ligated into pHa-1 expression vector prepared above that was previously linearized with Eco RI and Sma I. The 3' blunt end in MT created by the

25 Stu I digest was compatible with the blunt end in pHa-1 created by the Sma I digest. The resultant pUC18-derived vector containing a 5' _Drosophila_ MT gene fragment and a 3' ADH gene fragment was designated pRmHa-1. The pRmHa-1 expression vector contained the origin of replication (ori) and the beta-lactamase

30 gene conferring resistance to ampicillin (Amp^r) from pUC18 as shown in Figure 1A on the pHa-1 vector. The pRmHa-1 also contained from 5' to 3' the MT gene fragment, the multiple cloning site and the ADH gene fragment. The pRmHa-1 vector was

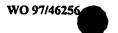used as described below in the construction of the pRmHa-3
expression vector.

        B.   <u>Preparation of pRmHa-2 Expression Vector</u>

5   For constructing the pRmHa-2 expression vector shown
in Figure 1A, the MT fragment prepared above was inserted into
the pUC18-derived vector pHa-1 as described for constructing
pRmHa-1 above with a few modifications. An Eco RI linker was
added to the Stu I site of the Eco RI/Stu I-isolated MT gene
10 fragment prepared above to form a metallothionein fragment
having Eco RI restriction sites on both ends. The resultant
fragment was then ligated into the ADH fragment-containing pUC18
expression vector that was previously linearized with Eco RI.
The resultant pUC18-derived vector containing a 5' Drosophila MT
15 gene fragment and a 3' ADH gene fragment having two Eco RI
restriction sites 5' to the multiple cloning site was designated
pRmHa-2. The pRmHa-2 expression vector contained the origin of
replication (ori) and the beta-lactamase gene conferring
resistance to ampicillin (Amp^r) from pUC18. The diagram of
20 pRmHa-2 also shows the 5' to 3' contiguous positions of the MT
gene fragment, the multiple cloning site and the ADH gene
fragment. The pRmHa-2 vector was used along with pRmHa-1 as
described below in the construction of the pRmHa-3 expression
vector.

25

        C.   <u>Preparation of pRmHa-3 Expression Vector</u>

            To prepare the pRmHa-3 expression vector that had only
one Eco RI restriction site, a fragment from pRmHa-2 was ligated
into pRmHa-1. For this construction, pRmHa-2, prepared above,
30 was first digested with Sph I. The resultant Sph I fragment
beginning in the middle of the MT gene and extending to the Sph
I site in the multiple cloning site was first isolated from the
pRmHa-2 vector and then ligated into pRmHa-1 that was previously

modified to remove the Eco RI restriction site 5' to the MT gene fragment then linearized with Sph I. This process is schematically illustrated in Figure 1B. To remove the Eco RI site in pRmHa-1, the vector was first digested with Eco RI to

5   form a linearized vector, then blunt ended with Mung Bean nuclease and religated.

A schematic of the pRmHa-3 vector is shown in Figure 1C. The relative positions of the various restriction sites from the pUC18 vector from which pRmHa-3 was derived are indicated in the

10 figure. The pRmHa-3 vector, being derived from pUC18, contains the pUC18 origin of replication and beta-lactamase gene conferring ampicillin resistance. Thus, MHC class II-encoding DNA fragments as prepared in this invention and cloned into the multiple cloning site of pRmHa-3 were transcriptionally

15 regulated by the MT promoter and polyadenylated via the ADH gene.

2.   <u>Preparation and Expression of Expressible MHC Class II Genes</u>

20   A.   <u>Amplification and Expression of MHC Class II Genes</u>

Genes or cDNAs encoding any preferred mammalian MHC Class II are cloned via use of the polymerase chain reaction (PCR). The primers described herein are used to amplify the appropriate Class II cDNAs in separate reactions which are then

25 cloned and sequenced. To create MHC class II proteins, full length murine IA^d α- and β- chain cDNAs were first obtained followed by PCR amplification and modification. The complete nucleotide sequences of the murine IA^d α-chain cDNA is described by Benoist et al., <u>Cell</u>, 34:169-177 (1983) and is also listed in

30 Genebank Accession Number K01923. The complete nucleotide sequences of the murine IA^d β-chain cDNA is described by Malissen et al., <u>Science</u>, 221:750-754 (1983) and is also listed in GenBank Accession Numbers K00007 and K00008. To amplify each

chain, mouse splenocytes were used as a source of total RNA.
First strand cDNA was synthesized by using oligo(dT) and avian
myeloblastosis virus reverse transcriptase. The resulting cDNA
was used in a PCR amplification reaction utilizing the

5 appropriate primers described below and a GeneAmp kit and
thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). Reaction
conditions preferably contained 1 μg cDNA template and 200 nM of
each oligonucleotide primer. Thirty cycles were run as follows:
(a) 1 minute at 92°C; (b) 1 minute at 60°C; and (c) 1 minute at

10 72°C. The PCR reaction was then heated to 99°C for 10 minutes
to inactivate the Taq polymerase and the ends of the DNA were
made blunt by T4 polymerase (Stratagene, La Jolla, CA).

For all the oligonucleotide primers indicated in the
Examples, the 5' primer is also referred to as the forward

15 primer or sense primer as it has the same sequence as the top
strand of the cDNA for hybridizing to the complementary bottom
strand. In contrast, the 3' primer is also referred to as the
backward primer or anti-sense primer as it has the same sequence
as the bottom strand of the cDNA for hybridizing to the

20 complementary top strand. Both primers are written in the 5' to
3' direction. The full length IA^d α-chain cDNA was amplified
with the 5' primer having the nucleotide sequence
5'CTTGAATTCCACCATGCCGTGCAGCAGAGCTCTGA3' (SEQ ID NO 3). The 5'
primer was also designed with an Eco RI restriction site to

25 allow for directional ligation of the amplified products into
recipient expression vectors. The 3' primer
5'TTTGGATCCTCATAAAGGCCCTGGGTGTC3' (SEQ ID NO 4) was also
designed to contain a Bam HI restriction site.

The full length IA^d β cDNA was amplified with the 5' forward

30 primer having the nucleotide sequence
5'CTTGAATTCCACCATGGCTCTGCAGATCCCCA3' (SEQ ID NO 5). The 5'
primer was also designed with an Eco RI restriction site to
allow for directional ligation of the amplified products into

recipient expression vectors. The 3' primer
5'TTTGGATCCTCACTGCAGGAGCCCTGCT3' (SEQ ID NO 6) was designed to
contain a Bam HI restriction site. The modified cDNAs encoding
the murine IA^d α- and β-chains were first separately

5   directionally cloned into the polylinker at the Eco RI and Bam
HI restriction sites of the metallothionein promoter-driven
pRmHa-3 vector, then sequenced by dye terminator technique on an
Applied Biosystem 373A automated sequencer.

The complete nucleotide sequences of the murine IA^d α- and

10   β-chain amplified regions cloned into pRmHa-3 are respectively
listed in SEQ ID NOs 7 and 8.

The separate plasmids were then transfected into _Drosophila
melanogaster_ Schneider-2 cells (ATCC CRL 10974, Rockville, MD)
as described elsewhere (Jackson et al., _Proc. Natl. Acad. Sci._

15   _U.S.A._, 89;12117-21 1992). Equal amounts of the plasmids
encoding the two class II chains were cotransfected together
with a neomycin resistance gene, plasmid phshsneo (Bunch et al.,
Nuc. Acids Res., 16:1043-1061 (1988) at a ratio of 1:30 to
produce stable cell lines that were derived by G418 selection in

20   Schneider's _Drosophila_ medium (Gibco/BRL, Grand Island, NY)
supplemented with 10% fetal calf serum (heat treated for 1 hour
at 55°C), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1
mM glutamine over a period of 4 weeks. Specifically, after
transfection, the supernatant was carefully removed and the

25   cells were transferred to a 75cm² flask in a total volume of 12
ml Schneider medium containing 500 μg/ml Geneticin (G418)
(Gibco/BRL, Grand Island, NY). After 4 days, 4 ml of the
culture were transferred to a fresh flask containing 6 ml of
Schneider medium with 500 μg/ml G418. This procedure was

30   repeated every 4-7 days until a stable population of cells
emerged which weakly adhered to the flask and grew with a
doubling time of approximately 24 hours. These cells were
subsequently cultured and passaged in the selection media as

described above.   Frozen aliquots of the stably transfected
cells were prepared by collecting 5-20 X 10⁶ cells by
centrifugation and resuspending them in 1 ml of cell freezing
media (93% fetal calf serum/7% dimethylsulfoxide).   Aliquots

5   were then placed at -70°C for 1 week and subsequently
transferred to liquid nitrogen storage. Expression of the
stably transfected MHC class II IAᵈ α- and β-chain genes was
induced with 0.7 mM cupric sulfate for 24 hours at 27°C.

Expression of MHC class II IAᵈ heterodimers at the cell

10   surface of the transfected Drosophila cells was evaluated by
flow cytometry after staining with with MKD6, an IAᵈ-specific
monoclonal antibody (Kappler et al., J. Exp. Med., 153:1198
(1981).   Briefly, aliquots of cells (5 X 10⁵) were transferred
into tubes on ice, collected by centrifugation (1,000 X g for 5

15   minutes), resuspended in 0.1 ml of Drosophila medium with 5%
horse serum containing the appropriate primary antibody (MKD6).
After a 20 minute incubation at room temperature, cells were
washed twice in 3 ml of Schneider's medium containing horse
serum and resuspended in 0.1 ml of medium containing FITC-

20   labeled secondary antibody (Cappell, Durham, NC).   After a 20
minute incubation on ice, cells were washed twice with
Schneider's medium containing horse serum and resuspended in
this buffer at a concentration of 1 X 10⁶/ml. Propidium iodide
was added to permit exclusion of dead cells from the analysis.

25   Samples were then analyzed by FACScan or FACSort instrument
(Becton Dickinson).   Synthetic antigen presenting cells of this
invention that express the murine IAᵈ MHC class II haplotype,
described herein and well known in the art, with one or more
accessory molecules were produced as described below.

30   Full-length human MHC class II α- and β-chains for the DR,
DQ and DP haplotypes are amplified from peripheral blood cells
which include B cells, macrophages and dendritic cells. The 5'

and 3' primers used for amplifying each of the haplotypes have
the following sequences:

DR α: 5' primer = 5'CCACCATGGCCATTAGTGGAGTC3' (SEQ ID NO 9)

           3' primer = 5'TTTGGATCCTTACAGAGGCCCCCTGCGTT3' (SEQ ID NO

5 10);

DR β: 5' primer = 5'CCACCATGGTGTGTCTGAGGCTCC3' (SEQ ID NO 11)

           3' primer = 5'TTTGGATCCTCAGCTCAGGAATCCTCTTG3' (SEQ ID NO

12);

DQ α: 5' primer = 5'CCACCATGGTCCTAAACAAAGCTCTGAT3' (SEQ ID NO

10 13)

           3' primer = 5'TTTGGATCCTCACAAGGGCCCTTGGTGTCT3' (SEQ ID NO

14);

DQ β: 5' primer = 5'CCACCATGGCTTGGAAGAAGGCCTTT3' (SEQ ID NO 15)

           3' primer = 5'TTTAGATCTCAGTGCAGAAGCCCTTT3' (SEQ ID NO

15 16);

DP α: 5' primer = 5'CCACCATGGGCCCTGAAGACAGAAT3' (SEQ ID NO 17)

           3' primer = 5'TTTGGATCCTCACAGGGTCCCCTGGGC3' (SEQ ID NO

18);

DP β: 5' primer = 5'CCACCATGGTTCTGCAGGTTTCTGCG3' (SEQ ID NO 19)

20   3' primer = 5'TTTGGATCCTTATGCAGATCCTCGTTGAA3' (SEQ ID NO

20).

The amplification conditions for obtaining the above human
haplotypes are identical to those described above for the murine
counterparts.

25   Synthetic antigen presenting cells of this invention that
express a human MHC class II haplotypes, described herein and
well known in the art, with one or more accessory molecules are
produced as described below.

30   B.   Amplification of Invariant and HLA-DM Chain cDNAs

    For some aspects of the present invention as described
in Section B in the detailed description, the invariant chain
and HLA-DM α and HLA-DM β are co-expressed with a selected MHC

class II heterodimer and at least one accessory molecule
described above. In this instance, the MHC class II α- and β-
chains, invariant chain and HLA-DM α- and β-chains are
transfected into recipient antigen presenting cells in molar

5   ratios 5:5:8:1:1. The ratio of accessory molecules to the molar
ratio of the other genes is 1:1. The resultant transfected
cells are then induced for expression of the exogenous genes as
described above to generate a synthetic antigen presenting cell
line of this invention.

10

1)   <u>Invariant Chain</u>

The murine invariant chain cDNA is generated from
mouse spleen cells. The full-length invariant chain is
amplified from this source with the primer pair of a 5' and 3'

15   primer having the respective nucleotide sequences
5'AAGAATTCACTAGAGGCTAGAGCCAT3' (SEQ ID NO 21) and
5'AAGGATCCTCACAGGGTGACTTGACC3' (SEQ ID NO 22). As described
above, the 5' and 3' primers were designed to respectively
incorporate Eco RI and Bam HI restriction sites. Following

20   cloning of the amplified invariant chain into a Eco RI/Bam HI-
digested pRmHa-3 vector, the resultant clone was sequenced and
determined to have the sequence listed in SEQ ID NO 23.

The human invariant chain cDNA used in this invention is
generated using RNA from γ-interferon-induced HeLa cells (ATCC

25   Accession Number CCL 2). The resultant cDNA is then used as a
template in PCR with the 5' and 3' oligonucleotide primers
having the respective nucleotide sequences
5'AAGAATTCACCATGGATGATCAGCGCGACCTT3' (SEQ ID NO 24) and
5'AAAGGATCCTCACATGGGGACTGGGCCCAGA3' (SEQ ID NO 25). The

30   resulting PCR fragments are cleaved with Eco RI and Bam HI
before ligation into similarly digested pRmHa-3.

2) HLA-DM

Messenger RNA from γ-interferon-induced HeLa cells is also used to synthesize the α- and β-chains of HLA DM cDNA that is then used as a template in PCR. The α-chain is

5   amplified with the 5' and 3' primer pair having the respective nucleotide sequences 5'AAACCATGGGTCATGAACAGAACCA3' (SEQ ID NO 26) and 5'TTTGTCGACTCAGTCACCTGAGCAAGG3'(SEQ ID NO 27). The β-chain is amplified with the 5' and 3' primer pair having the respective nucleotide sequences 5'AAACCATGGTCTCATTCCTGCC3' (SEQ

10   ID NO 28) and 5'TTTGTCGACCTAGGAAATGTGCCATCC3'(SEQ ID NO 29). The resulting PCR fragments are cleaved with Nco I and Sal I before separate ligation into a similarly digested pRmHa-3.

      C.   Amplification of Genes Encoding Accessory Molecules

15   1)   Adhesion Molecules

            a.   ICAM-1, ICAM-2, and ICAM-3

For isolating murine ICAM-1, spleen cells were isolated from Balb/c mice. The spleen cells were first stimulated with conA before isolation of mRNA using the

20   FastTrack kit (Invitrogen, San Diego, CA) according to the manufacturers' instructions. cDNA was synthesized from the mRNA as described above. The resultant cDNA was then subjected to PCR with the following respective 5' and 3' primers that were designed based on the published cDNA nucleotide sequence (Siu,

25   G. et al., J. Immunol., 143:3813-3820 (1989)):
5'TTTAGAATTCACCATGGCTTCAACCCGTGCCAAG3' (SEQ ID NO 30) and
5'TTTAGTCGACTCAGGGAGGTGGGGCTTGTCC3' (SEQ ID NO 31). The PCR products were then cleaved with the restriction enzymes Eco RI and Sal I and ligated into a similarly digested pRmHa-3.

30   The above expression construct was then co-transfected into Drosophila S2 cells with the murine IA^d α- and β-chain genes prepared above using the calcium phosphate method as described above in the molar ratio of 1:1:1 of ICAM-1:α-chain:β-chain.

Stably transfected cells were obtained as described above.
Synthetic antigen presenting <u>Drosophila</u> S2 cells expressing the
accessory molecule ICAM-1 with the IA^d α- and β-chains on the
surface of the cell were then produced by inducing expression as
5   previously described.

In other embodiments, synthetic antigen presenting cells
containing genes encoding ICAM-1, B7.1 and/or B7.2 in
conjunction with the murine IA^d molecules were also produced for
generating synthetic antigen presenting cells as used in
10   activation assays as described in Example 5.

Human ICAM-1 is similarly amplified from mRNA isolated from
the human cell line K562, originated from human chronic
myelogenous leukemia (ATCC Accession Number CCL-243) and
cultured under recommended conditions (i.e., RPMI with 10% fetal
15   calf serum at 37°C with 5% CO_2). The PCR primers for amplifying
the human accessory molecules are designed based on known
available sequences and in consideration of the 5' and 3'
cloning sites needed to clone into the appropriate vectors. The
nucleotide sequence of human ICAM-1 cDNA is available through
20   GenBank Accession Number GB J03132. The ICAM-1 5' and 3'
primers have the respective nucleotide sequences
5'ACCCTTGAATTCATGGCTCCCAGCAGCCCCCGGCCC3' (SEQ ID NO 32)   and
5'ATTACCGGATCCTCAGGGAGGCGTGGCTTGTGTGTTCGG3' (SEQ ID NO 33).

PCR is performed with these primers as previously described
25   to obtain amplified human ICAM-1. The resultant PCR products
are then cloned into pRmHa-3 as previously described followed by
transfection into recipient cells, also as previously described.

Similarly, human ICAM-2 and ICAM-3, the nucleotide
30   sequences of which are available with the respective GenBank
Accession Numbers GB X15606 and GB S50015, are amplifed with
comparable primer pairs. The respective 5' and 3' primers for
amplifying ICAM-2 have the nucleotide sequence

5'AAGGTACCCGTGGAGACTGCCAGAGAT3' (SEQ ID NO 34) and

5'TTTGGATCCCTATGGCCGGAAGGCCTG3' (SEQ ID NO 35). The respective

5' and 3' primers for amplifying ICAM-3 have the nucleotide

sequence 5'AAGAATTCCTGTCAGAATGGCCACCAT3' (SEQ ID NO 36) and

5   5'TTTAGATCTTCACTCAGCTCTGGACGGT' (SEQ ID NO 37). The resultant

amplified ICAM products are then separately ligated into pRmHa-3

and separately transfected into <u>Drosophila</u> S2 cells.

    Synthetic antigen presenting <u>Drosophila</u> S2 cells expressing

the accessory molecule ICAM-1 with a selected human MHC class II

10 haplotype on the surface of the cell are then produced by

inducing expression as previously described.

    In other embodiments, synthetic antigen presenting cells

containing genes encoding ICAM-1, ICAM-2 or ICAM-3 in

combination with B7.1 and/or B7.2 along with a human haplotype

15 are also produced for generating synthetic antigen presenting

cells as used in activation assays as described in Example 5.

Additional permutations contemplated for use in this invention

include the above combinations with LFA-3 and/or Fas ligand

(FasL), both of which are other accessory molecules as described

20 below.

         b.   <u>LFA-3</u>

    Human LFA-3 is isolated from blood

lymphocytes. The nucleotide sequence of human LFA-3 cDNA is

available through GenBank Accession Number GB I09083. Human

25 LFA-3 is amplified accordingly with a 5' and 3' primer having

the respective nucletide sequences

5'ACCCTTGAGCTCATGGTTGCTGGGAGCGACGCGGGG3' (SEQ ID NO 38) and

5'ATTACCGGATCCTTAAAGAACATTCATATACAGCACAATACA3' (SEQ ID NO 39).

    As described previously, synthetic antigen presenting cells

30 containing genes encoding human LFA-3 alone or in various

combinations with the other accessory molecules described herein

along with a human haplotype are also produced for generating

synthetic antigen presenting cells as used in activation assays
as described in Example 5.

>   2)   Costimulatory Molecules

>   a.   B7.1

5   cDNA was generated from mRNA isolated from
mice as described above for ICAM-1. The resultant cDNA was then
subjected to PCR with the following respective 5' and 3'
oligonucleotide primers shown in the 5' to 3' direction that
were designed based on the published cDNA nucleotide sequence
10 (Freeman, et al., J. Exp. Med., 174:625-631 (1991)):
5'TTTAGAATTCACCATGGCTTGCAATTGTCAGTTG3' (SEQ ID NO 40) and
5'TTTAGTCGACCTAAAGGAAGACGGTCTGTTC3' (SEQ ID NO 41). The PCR
products were cleaved with the restriction enzymes Eco RI and
Sal I and ligated into a similarly digested pRmHa-3.

15   The above expression construct was then co-transfected into
Drosophila S2 cells with the murine IA^d α- and β-chain genes
prepared above using the calcium phosphate method as described
above in the molar ratio of 1:1:1 of B7.1:α-chain:β-chain.
Stably transfected cells were obtained as described above.
20 Synthetic antigen presenting Drosophila S2 cells expressing the
accessory molecule B7.1 with the IA^d α- and β-chains on the
surface of the cell were then produced by inducing expression as
previously described.

         In other embodiments, synthetic antigen presenting cells
25 containing genes encoding murine ICAM-1, B7.1 and/or B7.2 in
conjunction with the murine IA^d molecules are also produced for
generated synthetic antigen presenting cells as used in
activation assays as described in Example 5.

         Human B7.1 is similarly isolated from K562 cells and cloned
30 into pRmHa-3 as described above. The nucleotide sequence of
human B7.1 cDNA is available through GenBank Accession Number GB
M83071. The 5' and 3' primers have the respective nucleotide
sequences, 5'ACCCTTGAATCCATGGGCCACACACGGAGGCAG3' (SEQ ID NO 42)

and 5'ATTACCGGATCCTTATACAGGGCGTACACTTTCCCTTCT3' (SEQ ID NO 43).

The resultant PCR products are then inserted into pRmHa-3 that

is then co-transfected along with amplified cloned human

haplotype genes into <u>Drosophila</u> S2 cells.

5   Synthetic antigen presenting <u>Drosophila</u> S2 cells expressing

the accessory molecule B7.1 with a selected human MHC class II

haplotype on the surface of the cell are then produced by

inducing expression as previously described.

          In other embodiments, synthetic antigen presenting cells

10   containing genes encoding human B7.1 in various combinations

with the other accessory molecules described herein along with a

human haplotype are also produced for generating synthetic

antigen presenting cells as used in activation assays as

described in Example 5.

15

          b.   <u>B7.2</u>

          Murine IC-21 cells (ATCC Accession Number

TIB 186) were propagated in RPMI 1640 medium containing 10%

fetal calf serum. cDNA was synthesized from the mRNA isolated

20   from these cells as described above. The resultant cDNA was

then subjected to PCR with the following respective 5' and 3'

oligonucleotide primers shown in the 5' to 3' direction that

were designed based on the published cDNA nucleotide sequence

(Freeman, et al., <u>J. Exp. Med.</u>, 178:2185-2192 (1993)):

25   5'TTTAGAATTCACCATGGACCCCAGATGCACCATGGG3' (SEQ ID NO 44) and

5'TTTAGTCGACTCACTCTGCATTTGGTTTTGCTGA3' (SEQ ID NO 45). The PCR

products were cleaved with the restriction enzymes Eco RI and

Sal I and ligated into a similarly digested pRmHa-3.

          The above expression construct was then co-transfected into

30   <u>Drosophila</u> S2 cells with the murine IA^d α- and β-chain genes

prepared above using the calcium phosphate method as described

above in the molar ratio of 1:1:1 of B7.2:α-chain:β-chain.

Stably transfected cells were obtained as described above.

Synthetic antigen presenting <u>Drosophila</u> S2 cells expressing the
accessory molecule B7.2 with the IA^d α- and β-chains on the
surface of the cell were then produced by inducing expression as
previously described.

5   In other embodiments, synthetic antigen presenting cells
containing genes encoding murine B7.2 in conjunction with ICAM-1
and the murine IA^d molecules were also produced following the
procedures described above for generated synthetic antigen
presenting cells as used in activation assays as described in
10 Example 5.

Human B7.2 is isolated from the human cell line HL60, that
originated from a human promyelocytic leukemia (ATCC Accession
Number CCL-240). The nucleotide sequence of human B7.2 cDNA is
available through GenBank Accession Number GB M83071. The 5'
15 and 3' primers for amplifying human B7.2 have the respective
nucleotide sequences 5'ACCCTTGAGCTCATGGATCCCCAGTGCACTATG3' (SEQ
ID NO 46) and 5'ATTACCCCCGGGTTAAAAACATGTATCACTTTTGTCGCATGA3'
(SEQ ID NO 47).

The amplified B7.2 products are then cloned into pRmHa-3 as
20 previously described for tranfection into <u>Drosophila</u> S2 cells
along with constructs for a selected human haplotype.
Expression of the transfected genes is induced as previously
described.

In other embodiments, synthetic antigen presenting cells
25 containing genes encoding human B7.2 in various combinations
with the other accessory molecules described herein along with a
human haplotype are also produced for generating synthetic
antigen presenting cells as used in activation assays as
described in Example 5.

30

    3.   <u>Survival Molecules</u>

Human Fas ligand is isolated from activated human
T cells. The nucleotide sequence of human Fas ligand cDNA is

available through GenBank Accession Number GB U08137. Human Fas
ligand is amplified accordingly with a 5' and 3' primer pair
having the respective nucleotide sequences
5'AAAGGATCCACCATGCAGCAGCCCTTCAATT3' (SEQ ID NO 48) and

5   5'TTTGGATCCTTAGAGCTTATATAAGCCGA3' (SEQ ID NO 49).

     Human CD70, the ligand for CD27 expressed on T cells, is
amplified as described above with a 5' and 3' primer pair having
the respective nucleotide sequences
5'AAAGAATTCGGTACCATGCCGGAGGAGGGTTCGG3' (SEQ ID NO 50) and

10   5'TTTGGATCCTCAGGGGCGCACCCACTGCA3' (SEQ ID NO 51).

     As described previously, synthetic antigen presenting cells
containing genes encoding human Fas ligand alone or in various
combinations with the other accessory molecules described herein
along with a human haplotype are also produced for generating

15 synthetic antigen presenting cells as used in activation assays
as described in Example 5.

3.   <u>Preparation of MHC Class II Anchored on Synthetic
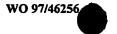   Supports</u>

20   The examples described herein present a new method to
immobilize high amounts of MHC class II molecules and single
accessory molecules or various combinations thereof on various
surfaces (fly cells, red blood cells, latex beads) in native
conformation as judged by monoclonal antibody binding and

25 rosetting experiments (T cell receptor binding). This method
can be extended to other synthetic surfaces including artificial
phospholipid membranes. Phosphatidylethanolamine as well as
avidin-coupled phospholipids are particularly relevant this
invention. These phospholipids are commercially available from

30 Lipex Biomembrane Inc., Vancouver, BC, Canada.

A.   Immobilization of Biotinylated MHC Class II on
      Avidin-Coated Red Blood Cells

         NHS-LC-biotin, neutravidin and biotin-BMCC are
purchased from Pierce (Rockford, IL). Sheep red blood cells are
5   obtained from the Colorado Serum Company (Denver, CO).
Drosophila S2 cells expressing MHC c.lass II are prepared as
described above. Monoclonal antibodies MKD6 and M5-114 (ATCC
Accession Number TIB 120 for the hybridoma secreting MKD6) are
used as hybridoma cell culture supernatants. The protocol used
10   is described by Muzykantov and Taylor (Anal. Biochem.,
223:142-148 (1994)). Briefly, SRBC are washed four times in
PBS, biotinylated using NHS-LC-biotin, washed again 4 times in
PBS, incubated with neutavidin, and finally washed four times
and stored at 4°C in PBS containing 3% fetal calf serum and
15   0.02% sodium azide. Recombinant expressed MHC class II α- and
β-chains are biotinylated using biotin-BMCC, a maleimide-coupled
biotin which reacts with thiol groups. Biotinylation is
performed as recommended by the manufacturer. Unreacted biotin
is removed using Centricon 10.

20   Biotinylated MHC class II α- and β-chains are immobilized
by incubation at a final concentration of 0.2 mg/ml with
avidin-coated SRBC for 30 minutes followed by washing in DMEM
containing 10% fetal calf serum. SRBC with attached MHC class
II are used immediately.

25   Immobilization of biotinylated MHC class II α- and β-chains
on avidin-coated SRBC is done as indicated above. Attachment is
assessed using flow cytofluorometry using the antibodies
described above. For rosetting assays, either Drosophila S2
cells expressing MHC class II or MHC class II-coated SRBC are
30   incubated with specific peptide (see Example 4) (0.02 mM) or an
irrelevant peptide (0.02 mM) for 30 minutes on ice; CD4+ T cells
are then added, the proportion being 10 T cells for one
Drosophila S2 cell, or 10 SRBC for 1 T cell. The mixture is

then pelleted and kept on ice for at least 30 minutes. Cells

are then carefully resuspended and rosettes are counted, a

rosette being a Drosophila S2 cell bound to at least 3 CD4+ T

cells, or a CD4+ T cell bound to at least 3 SRBC.

5

    B.   Immobilization of Biotinylated MHC Class II on

          Avidin-Coated Latex Beads

          Six micron diameter latex sulfate beads are purchased

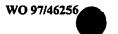from Interfacial Dynamics Corporation (Portland, OR) and

10 biotinylated according to the protocol described above.

Avidin-coated latex beads are prepared using a 1% suspension of

the latex beads incubated in PBS containing 1 mg/ml of

neutravidin for 1 hour at room temperature. An equal volume of

PBS containing 10% fetal calf serum is then added. After 1 hour

15 of incubation at room temperature, the beads are washed 3 times

and used for binding of recombinant biotinylated MHC class II.

          Recombinant biotinylated MHC class II is immobilized by

incubation at a final concentration of 0.2 mg/ml with

avidin-coated latex beads for 30 minutes followed by washing in

20 DMEM containing 10% fetal calf serum. SRBC with attached MHC

class II are used immediately. Rosetting experiments are

performed as previously desribed.

    C.   Immobilization and Detection of MHC Class II

25   Bound to Various Solid Supports Such as Plastic

         Microwell Plates

          The MHC class II molecules are immobilized by direct

binding to microtiter plates (Corning) and detected as follows:

MHC class II is diluted to a desired concentration in PBS, e.g.

30 1 mg/ml for 100 ng/well, and then added to each well on the

plastic microtiter plate. The plate is incubated for 1 hour at

room temperature. After incubation, the plate is washed once

with PBS and 200 μl 2% bovine serum albumin (BSA) in PBS +

(.05%) and Tween (PBST) is added followed by incubation for another hour at room temperature. The plate is washed 3 times with PBST and biotinylated anti-MHC class II monoclonal antibody was added (1:2500) in 2% BSA in PBS. The plate is incubated

5   another hour at room temperature and washed 3 times with PBST. Avidin conjugated HRP is added (1:2500) in 2% BSA in PBS. Following another hour of incubation at room temperature, the plate is washed 3 times with PBST and H_2O_2 or thophenyldiamine was added. The reaction is stopped with H_2SO_4. Reaction product

10   is detected colorimetrically at 490 nm. Recombinant MHC class II molecules can alternatively be bound through biotin-avidin linked interactions with the substrate. In this embodiment, the microwell plates are coated with 100 μl avidin diluted in PBS to a concentration of 0.001 mg/ml. Excess avidin is removed by a

15   PBS wash. The above procedure for presenting and detecting MHC class II binding is followed.

Recombinant MHC molecules are alternatively immobilized by a linkage based on a poly-histidine tag added to the MHC interacting with the nickel bound to the substrate.

20   The above procedure for binding and detection is followed using nickel chelate coated microwell plates (Xenopore) and expressed recombinant MHC molecules with a poly-histidine tag.

4.   Peptide Generation

25   Antigenic peptides according to the present invention are obtained from naturally-occurring sources, including those described in the specification, or may be synthesized using known methods. In various examples disclosed herein, all peptides were separately chemically synthesized by T-BOC

30   chemistry on an Applied Biosystem instrument, ABI 431A (Foster City, CA), and purified on a C18 reverse phase column. Isolation or synthesis of peptides generated from a random assortment of amino acid residues may also be appropriate,

particularly when one is attempting to ascertain a particular epitope in order to load an empty MHC molecule with a peptide most likely to stimulate CD4 cells. Mixtures of randomized peptides are obtained by using proteasomes, by subjecting a

5   protein or polypeptide to a degradative process, such as digestion with chymotrypsin, or by synthesis.

While the cell lines of the present invention are able to degrade proteins and polypeptides into smaller peptides capable of being loaded onto human MHC class II molecules, it is

10 preferable to introduce the peptides directly into the cell culture to facilitate a more rapid loading and expression process.

If peptides are synthesized having randomly incorporated amino acid residues, all varieties of amino acids, L or D

15 conformations as well as modified amino acids, are preferably incorporated during each cycle of the synthesis. It should be noted, however, that various parameters, e.g., solvent incompatibility of certain amino acids, may result in a mixture which contains peptides lacking certain amino acids. The

20 process should thus be adjusted as needed, by altering solvents and reaction conditions, to produce the greatest variety of peptides. A peptide used for peptide loading as described in Example 5 with murine MHC class II is ovalbumin$_{323-339}$ (OVA) having the amino acid residue sequence ISQAVHAAHAEINEAGR (SEQ ID NO

25 52). Preferred peptides specific for human MHC class II haplotypes include the following peptides: 1) Influenza hemagglutinin$_{306-318}$ PKYVKQNTLKLAT (SEQ ID NO 53) that binds HLA-DRB1*0101; 2) HSP65$_{2-12}$ from M. tuberculosis KTIATDEEARR (SEQ ID NO 54) that binds HLA-DRB1*0301; 3) Mitochondrial outer protein

30 (MOMP) of C. trachomatis QASLALSYRLNMFTP (SEQ ID NO 55) that binds HLA-DRB1*0301; and 4) HLA-A2$_{33-45}$ FVRFDSDAASQRM (SEQ ID NO 56) that binds to HLA-DRB1*0401. An extensive listing of known class II-binding peptides as well as a description of how

peptide ligands are defined is presented by Rammensee et al.,
Immunogenetics, 41:178-228 (1995). Peptides for use in
practicing the methods of this invention are also available
through Chiron Mimetopes. In other embodiments, peptide
5   libraries, readily produced by one of ordinary skill in the art,
are contemplated for use in practicing the methods of this
invention with the synthetic antigen presenting cells as
described herein.

10   5.   Loading of Empty MHC Class II Molecules by In Vitro
          Incubation with Peptides

          As shown by the results presented below, the murine MHC
class II molecules along with selected accessory molecules
expressed on the surface of _Drosophila_ cell lines, having been
15   transfected with various combinations of plasmids encoding MHC
class II and accessory molecules, bind specific peptides in a
dose-dependent fashion and are able to present these peptides to
antigen specific T cells resulting in T cell activation.

          For the proliferation and activation assays performed
20   below, _Drosophila melanogaster_ Schneider 2 (SC2) cells (ATCC
Accession Number CRL 10974) were transformed with genes encoding
the MHC class II α- and β-chain from H2-A^d as described in
Example 2. For other assays where selected accessory cells were
expressed in combination with the MHC class II molecules, the
25   genes for B7.1, B7.2 and ICAM-1, were also introduced into the
MHC gene-containing cells as previously described.

          The accessory molecules were expressed at selected
concentrations and in combinations as defined below to permit
reproducible and consistent activation of CD4$^+$ cells. Following
30   expression, induced by treatment with copper sulfate, the
concentrations of expressed molecules were monitored. Selection
of cells expressing the desired levels of molecules was
performed with flow cytometry sorting of cell lines. Table I

84

lists the <u>Drosophila</u> cell lines expressing murine IA^d MHC class
II molecules alone or in combination with various accessory
molecules. The table also presents a summary of the results of
proliferative effects, described further below, as indicated

5   with a "-" for no effect and a "+" for a positive proliferative
effect. A summary of the types of cytokines produced by each
type of synthetic antigen presenting cell is also indicated, and
described further below, with the additional notation with
arrows of whether a particular cytokine production was up or

10   down regulated.

                              Table 1

     <u>Accessory</u>   <u>Proliferative</u>   <u>Cytokines</u>
     <u>Molecules</u>   <u>Responses</u>   <u>Produced</u>

15   None   -   -

     B7.1   +   IL-4, IL-10, IL-2

     B7.2   +   IL-4, IL-10, IL-2

     ICAM-1   -   -

     B7.1 + B7.2   +   IL-4, IL-10, IL-2

20   B7.1 + ICAM-1   +   ↑IL-2, ↓IL-4, ↓IL-10

     B7.2 + ICAM-1   +   ↑IL-2, ↓IL-4, ↓IL-10

     CD70 + ICAM-1   +   IL-2

     CD70   -   -

     CD70 + ICAM-1

25   + B7.2   +   ↑IL-2

     For assessing the effect of antigen presentation and
ultimately T cell response, the antigen specific T cells were
CD4$^+$ cells obtained from T cell receptor transgenic mice. The

30   transgenic line as used herein was D01 which expresses a T cell
receptor specific for the ovalbumin peptide 323-339. The D01
mouse line is obtained from Dr. Dennis Lo. For assaying T cell
responsiveness to the recombinant surface-expressed MHC class II

molecules, CD4⁺ cells were first purified from lymph nodes using a combination of techniques designed to eliminate all host APC from the responder population. Small numbers (5 X 10⁴) of these highly purified CD4⁺ cells were then cultured with the _Drosophila_

5   cell lines in the presence or absence of ova peptide.

The effect of peptide presentation in the presence of MHC class II with and without accessory molecules, B7.1, B7.2 or B7.2 plus ICAM-1, was first assessed in a proliferation assay performed as described by Webb et al., _Cell_, 63:1249 (1990).

10 Briefly, 5 X 10⁴ CD4⁺ T cells were cultured in microtiter wells with 5 X 10⁴ _Drosophila_ cells in 300 μl of culture medium. Eighteen hours prior to harvesting, 100 μl of supernatant were removed for cytokine analysis and 1 μCi of ³H-thymidine was added to each well. At the times indicated (3, 4 or 5 days), the

15 wells were individually harvested onto glass fiber filters and counted in a liquid scintillation counter.

As seen in Figure 2, cell lines expressing MHC class II molecules alone failed to stimulate T cell proliferative responses as indicated by the low incorporation of radioactive

20 thymidine; higher counts indicate greater proliferation plotted against the amount of stimulating peptide in μM. However, co-expression of either B7.1 or B7.2 costimulatory molecules conferred the capacity of MHC class II presented-ova peptide to stimulate strong proliferative responses. For comparison, the

25 results with splenic APC (labeled T-S) are shown in Figure 2.

As shown in Figure 3, _Drosophila_ cell lines expressing MHC class II molecules with the adhesion molecule ICAM-1 were nonstimulatory. However, the expression of ICAM-1 together with B7.2 generated a synthetic APC cell line that stimulated

30 proliferative responses at lower concentrations of peptide antigen. These results demonstrate that _Drosophila_ cell lines expressing the murine H2-Aᵈ MHC class II molecules and costimulatory molecules, B7.1, B7.2 and B7.2 plus ICAM-1, are

able to activate naive CD4⁺ T cells as measured by an increase in proliferation.

In addition to proliferation, the reproducible activation of CD4⁺ T cells to produce particular cytokines was desired.

5   Thus, the cytokines produced in the cultures described above were also evaluated using standard ELISA methods with antibodies specific for the cytokine to be measured. The data plotted in Figures 4A-4D and 5A-5D indicate the amount of cytokine produced in ng/ml concentrations against the stimulatory molecules on

10   APC.

The results of these assays indicate that different combinations of accessory molecules on Drosophila APC expressing recombinant MHC class II leads to the production of different cytokines as shown in Figures 4A-4D and Figures 5A-5D. For IL-2

15   production, a Th1 type response, cell lines expressing MHC class II with B7.1 or B7.2 were poorly stimulatory as shown in Figure 4A. In order to achieve significant levels of IL-2, the Drosophila cell lines required co-expression of ICAM-1, B7 and class II (Figure 5A). Production of γ-interferon was also

20   poorly induced by Drosophila cell lines expressing B7.1 and B7.2 (Figure 4D). In contrast to the results with IL-2, however, the co-expression of ICAM-1 did not restore γ-IFN production (Figure 5C). Splenic APC (labeled as T-S) induced strong γ-IFN production suggesting that additional molecules regulate the

25   production of this cytokine.

IL-4 production, a Th2 type response, is generally not observed during primary stimulation of naive CD4⁺ T cells. In keeping with these observations, splenic APC (T-S) failed to induce significant IL-4 after culture with D01 cells and

30   ova-peptide (Figure 5B). However, Drosophila cells co-expressing class II MHC molecules with either B7.1 or B7.2 or both induced strong IL-4 production during culture (Figures 4B and 5B). Interestingly, the addition of ICAM-1 had an

antagonistic influence on the production of this cytokine (Figure 5B). IL-10 was similarly regulated to IL-4 as shown in Figures 4D and 5D.

As described above, the results with a cytokine profile of a Th2 type response, rather than a Th1 type response, were obtained with cell lines expressing B7 and MHC class II. In contrast, co-expression of MHC class II in combination with B7 and ICAM-1 antagonized the production of IL-4 and IL-10 while strongly promoting IL-2, thereby inducing a Th1 type response with support the present invention in the ability to drive the activation of CD4$^+$ T cells into desired T cell subsets. These results definitively show that _Drosophila_ cell lines can be tailored to express defined combinations of accessory molecules for use in achieving a desired population of CD4$^+$ T cells that produce different patterns of cytokines. Thus, the compositions and methods described herein validate the compositions and methods of use of the present invention.

The foregoing is intended to be illustrative of the present invention, but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the invention.